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PREFACE

The addition of a preface to a volume already adorned with an introductory chapter may seem to be a gilding of the lily. We write these prefaces from a habit that is not easily broken after 23 years of indulgence, from the sweet realization that there is no super editor to cavil at the excursions of our pen, and from the possibly erroneous belief that volumes of this sort are unacceptable unless overlaid with an editorial contribution.

In point of fact these intrusions upon the reader are intended to accomplish only two objectives: to mention anything concerning content, editorial policy, and plans that may be of general interest; and to express publicly our gratitude to the authors of the review. We believe that their efforts are widely appreciated, especially by that growing company of scientists who themselves have suffered through the experience of writing reviews. The papers to be included in the review must be selected with utmost care, their contents must be weighed and appraised, the essay must be written, and the orchestration must not be forgotten.

While intrusion, upon author and reader alike, is the time-honored prerogative of editors, the line that separates helpfulness from obnoxiousness is extremely thin. The authors of these reviews are not infrequently more experienced, mature, and competent in the use of the Queen's English than those of us who tamper with their manuscripts. And even were editors cursed with infallibility it should still be the privilege of a mature writer to hang himself with a rope woven from his own mistakes. We recognize the weight of this argument, and we respect the eminence of many of its proponents. However, we also believe that if a house is to be kept in order it must have its rules. Our own house rules tend, with the passing of the years, to become more and more numerous and perhaps more and more trivial. They deal with the comma, the hyphen, and the full stop, the overloaded sentence, the dangling participle, the bibliography, the references in the text, abbreviations, jargon, figures, and formulae. Jargon and obscure abbreviations into which we moderns retreat with greater and greater frequency are fast becoming the bane of our existence. We commend to the attention of the reader an excellent little article by Elder, "Jargon—Good and Bad" [Science, 119, 536 (April 23, 1954)] and, while at it, two other articles in the same number: Struck, "Recommended Diet for Padded Writing" [Science, 119, 522 (1954)]; McCartney, "Does Writing Make an Exact Man?" [Science, 119, 525 (1954)]. Two books by Eric Partridge: "You Have a Point There" (a guide to punctuation), and "English, a Course for Human Beings," are both entertaining and useful. Hamish Hamilton (London) is the publisher.

We note, with pleasure, that all of the reviews announced for inclusion in the present volume were received. The section on water-soluble vitamins

has been expanded by division into two parts and will be further extended in 1955 into three parts. This division into three sections will be continued so long as interest in the subject continues to be so well-sustained. If the volume appears thin, in comparison with some of its predecessors, it is a passing phase: volume 24, with twenty-two chapters, will be obese and also meaty.

We wish to thank our editorial assistants and their associates in the business office, all of whom have given us their devoted help in the production and distribution of the Reviews. Mrs. Lillian Rutherford has been the principal editorial assistant for this present volume, and Professor Clark Griffin has given us his very generous aid in preparing the subject index.

As always, we are indebted to our printers, the George Banta Publishing Company, for their wholehearted co-operation.

H.J.A.	T.H.J.
A.K.B.	H.S.L.
H.J.D.	J.M.L.
W.Z.H.	G.M.

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ERRATA

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- page 20, Scheme B: for acetyl— $\overline{\text{S}}$ —SH read acetyl— $\overline{\text{S}}$ —SH
- page 28, line 36: for adeninen read adenine
- page 29, line 17: for dihydroxyactonephosphate read dihydroxyacetone-phosphate
- page 52, reference 46: for Mahler, H. R., Sarkar, N. K., and Alberty, R. A. read Mahler, H. R., Vernon, L. P., Sarkar, N. K., and Alberty, R. A.
- page 60, lines 10 through 14: for If two monovalent ions are brought from the distance a to the distance b (Å) in water the heat of reaction is about $-4(a-b)/ab$ kcal., and the value found by Dobry & Sturtevant may therefore also be explained qualitatively on this electrostatic basis especially since the reaction is hardly complete in the pH-range studied by them. read A similar explanation may be applied to the results of Dobry & Sturtevant, but probably only if the ions come very close to each other.
- page 111, lines 34 and 35: for 1,2-isopropylidene-D-xyloglutaric diadehyde read 1,2-isopropylidene-D-xylo-trihydroxyglutaric dialdehyde
- page 111, line 44: for lithium hydride read lithium aluminum hydride
- page 394, reference 5: for 15 read 16
- page 490, references 107 through 110: for Grove, F. G. read Grove, J. F.
- page 580, line 27: for pantetheine read pantothenic acid
- page 605, line 38: for [Nagaya (588)] read [Ata, Ito & Iso (588)]
- page 627, reference 588: for Nagaya, T., Japan J. Med Sci., Biochem., [II] read Ata, S., Ito, I., and Iso, N., Nagoya J. Med Sci.,
- page 655, reference 209: for (1591) read (1951)

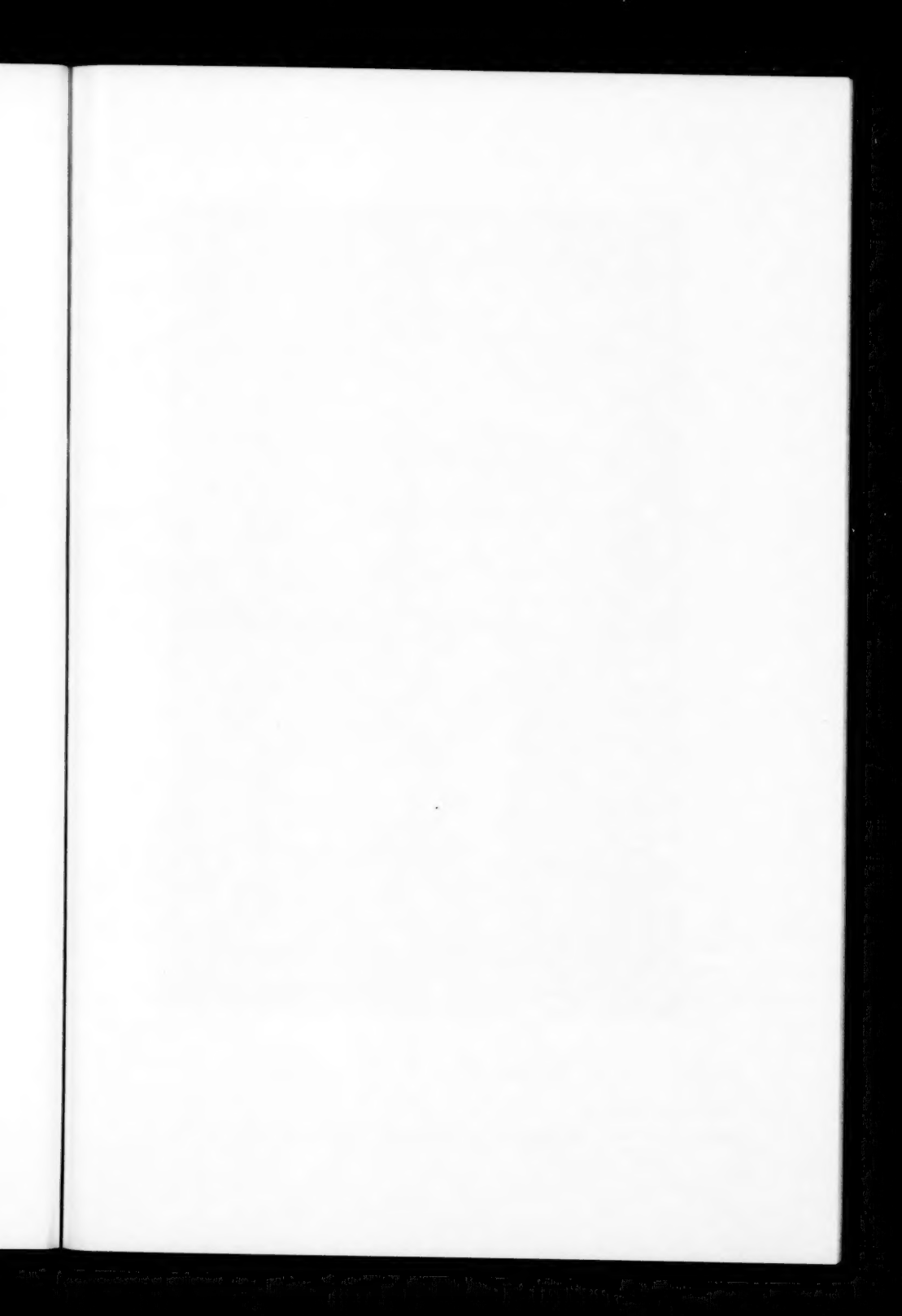
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- page 679: insert Ata, S., 605
- page 690: delete Grove, F. G.
- page 690: insert Grove, J. F., 377, 382, 471, 472, 473, 474
- page 693: insert Iso, N., 605
- page 693: insert Ito, I., 605

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Carl Freund
Sökösen, November, 1953.

PREFATORY CHAPTER

FIFTY YEARS OF BIOCHEMISTRY IN GERMANY

BY KARL THOMAS

Max Planck Gesellschaft zur Förderung der Wissenschaften, Göttingen, Germany

In the previous *Annual Review of Biochemistry* Professor E. V. McCollum who had received his basic training in chemistry presented his story of how he became a nutritionist. I, too, did not pursue my career in medicine which I first studied but turned to biochemistry and the investigation of nutritional problems.

PROFESSIONAL CAREER

I grew up in Freiburg i. Br. (Baden) where my father was a university professor of internal medicine and pediatrics. As a grammar-school boy, I had an opportunity to scan through medical journals in my father's house and, as a result, made up my mind about my future profession very early in life. I remember two events which I believe induced me to study medicine. One day when my father and I went for a walk, one of my father's colleagues, Eugen Baumann, joined us. He was the man who a short while later discovered iodine in the thyroid gland. At home we often talked about this discovery and its effect on the need for goiter surgery. We wondered whether the endemic cretinism occurring in the surroundings of my native town could finally be cured. The other event goes back to 1896. Professor Zehnder, a friend and co-worker of Röntgen, gave an experimental lecture about the new radiation. Sitting next to my father in the front row, I was asked to step in front of the screen to be observed as an object of a scientific experiment. Thus, as a boy of 13, I excitedly saw the beating of my own heart projected on the screen.

Many professions are chosen without proper conceptions. Today I ask myself why I really wanted to become a physician and not a chemist. Frankly I had no idea about the profession of a chemist since there was no chemical industry in Freiburg at that time, nor did we have much contact with industry of any kind in my parents' home. However, as a student, I twice heard outstanding series of chemical lectures, one given by Kiliani and the other by the famous Gattermann. Chemistry was not mandatory in my medical school curriculum; it was my keen interest in the subject that prompted my attendance at these lectures. During my student days the experimental lectures given by the physiologist, J. von Kries, proved to be the most interesting. Von Kries was a pupil of Karl Ludwig (Leipzig) and had become professor at Freiburg very early in his career. The text book by Bunge who taught in the nearby city of Basel left a lasting impression on me. My analytical work was completed under the supervision of Authenriet who invented the Hellige-colorimeter. In 1906 I finished my thesis on "The Color Reactions of Urobilinogen" in the laboratory of the hospital, pursuing the work under

rather primitive conditions. My thesis, while interesting, did not produce much of value. However, I received my M. D. degree at Freiburg in 1906. Some months later Professor Thierfelder offered me a position as an assistant in the newly erected Hygiene Institute of the University of Berlin under Max Rubner. Thus, I started my career as a nutritionist. Rubner, the head of this modern, roomy, and well-lighted institute, was very liberal, and we enjoyed a great deal of freedom in our work.

During the fall of 1907 an international congress for Hygiene and Demography took place in Berlin under the presidency of Rubner. Rubner himself presented several papers, among them one about the nitrogen minimum in human nutrition. As assistants, we received assignments to write about the congress in the technical journals and for the daily press, a welcome task to increase our really very small income.¹ Soon I found myself involved in a self-experiment to determine the nitrogen minimum and the biological value of proteins. These experiments aroused Rubner's personal interest, and he retained me as an assistant when he became director of the physiological institute of the University of Berlin in 1909. The following year I was drafted into the army for one year. After that, I asked Rubner for a leave of absence to round out my knowledge of chemistry. Unfortunately, I soon found out at Tübingen that one year was too short a time for this purpose and that a second year would certainly be necessary; to this Rubner reluctantly agreed. Thus, in 1911 I became an assistant in the Physiological-chemistry Institute in Tübingen under Professor Hans Thierfelder. I am still very grateful to my teacher, Thierfelder, who from the professional as well as from the human point of view taught me much. At that time Thierfelder was studying the lipids of the brain and, together with one of his Ph. D. candidates, K. Lock, I was allowed to assist in determining the amino acids of the blood plasma protein. Since the institute belonged to the Faculty of Natural Sciences I, as a medical man, was not eligible for a professorship there. For this reason I moved to the Physiological Institute in Greifswald where under Max Bleibtreu I became "Privatdozent" in the medical faculty. However, I soon returned to Rubner in Berlin.

On the occasion of the centenary of the University of Berlin in 1911 the Kaiser Wilhelm Gesellschaft zur Förderung der Wissenschaften was founded. This was a research organization with laboratories spread all over Germany. The society sponsored research in various branches of science, particularly in medicine. In 1913 I became a staff member at the Kaiser Wilhelm Institut für Arbeitsphysiologie which had an annual budget of about 40,000 marks (\$10,000). This was the finest, most carefree, and in a certain way, the most productive time of my life. A small new building was erected according to my own plans in the vicinity of the Physiological Institute. Just when the building was completed in August, 1914, the first

¹ In those days the Prussian state was very economically minded. An assistant got 100 marks (\$25) per month with an additional allowance of 25 marks for Berlin residents.

World War began. On the second day of the war I was in uniform to serve as a physician. At the end of 1915 I was severely wounded but was able to resume research in Berlin by the end of 1916. Then came the years of famine—1916 to 1919, and, with the end of the war, revolution and inflation of the German currency. Anyone who has not experienced this period personally can ever conceive the destructive effects which this liquidation of all property and possessions had on all levels of society as well as on the structure of the government. In the midst of these communistic upheavals in central Germany, caused by the economic crisis, I became professor of physiological chemistry at the Sächsische Landes Universität of Leipzig. Hans Fischer and F. Knoop had both declined this offer because they considered the institute too small. I accepted the appointment in the hope that enlargements and improvement of the facilities would be made in due time. I gradually became successful in my efforts, particularly after I refused two offers, one to Freiburg in 1928 and one to Basel in 1932. The condition for staying was that the expansion program in Leipzig would be expedited both in regard to space and budget. Yet, in spite of all my personal efforts, I obtained only an annex to the existing building rather than new and more modern quarters. This was finished in the fall of 1939 shortly after another war started and some of my co-workers were again drafted and had to leave. We continued our work under difficult conditions until Saxony was conquered by the American Army and subsequently ceded to the Russians; I was taken to the Western Zone by the American occupation forces. I succeeded in getting reestablished at the Bavarian University of Erlangen where I was appointed dean of the medical faculty. In the meantime, headquarters of the Kaiser Wilhelm Gesellschaft was moved from Berlin to Göttingen, and I was offered the directorship of the newly founded medical research institute and the management of its biochemical department. Soon after, the name of the society was changed to "Max Planck Gesellschaft." So for the fourth time in my life I had to begin anew to build an institute and provide the necessary facilities for experimental work.

The non-German reader of these lines is probably unaware of the difficult external conditions under which my generation had to work, particularly in the field of science and research. Politics interfered with our lives too many times. Even those of us who had little personal interest in politics could not always escape being drawn into the whirlpool. I, for one, was guided throughout my career, in my decisions as a research man and physician, by my own conscience and not by political expediency. Some of my decisions, particularly after 1933, required some moral courage for which I had to pay. However, in the last analysis I realize that a scientist may compare himself with the man of the sea, for it is only in stormy weather that one can prove himself to be a good sailor.

SCIENTIFIC WORK

Nitrogen-minimum.—After Rubner's presentation at the International Hygiene Congress of his work on the nitrogen minimum, it occurred to

me that in all previous work on nutrition, protein was computed as $6.25 \times \text{gm. N}$ but was never isolated; moreover that the differences in composition of the nitrogen-containing amino acids of food were not sufficiently considered. Could it be possible then that the different amino acids possessed different biological values? This I had to find out. First I checked older literature references experimentally. Subsequently, in a self-experiment, I determined my own nitrogen minimum with potatoes, bread, during starvation, as well as with an ample carbohydrate diet free of nitrogen. The latter consisted of a cold slurry made of cane sugar and starch taken five times a day. It is obvious that a "food" like this can be consumed in a self-experiment only for a certain length of time; even dogs will not accept this kind of "food". This was in 1907 about the time when McCollum began to explore similar problems with experiments on rats. However, I only learned this from the literature much later.

I found that on this pure carbohydrate diet I could reach a nitrogen minimum lower than all values previously cited in the literature. Furthermore, the daily urinary excretion of nitrogen remained fairly constant. This result was particularly significant since this nitrogen value became my reference standard for a true nitrogen minimum. I concluded that the lowest level of protein intake at which nitrogen equilibrium could be maintained just had to be equal to the quantity of the nitrogen-containing compounds taken from the body in case of a calorically abundant supply of sugar.

Rubner did not pay much attention to my work for quite awhile. The "*Geheimrat*" was considered to be a rather difficult person and not easily accessible. However, one morning when neither one of his two co-workers, Dr. Kisskalt (now a retired professor in Munich) nor Dr. Nawiasky (now a well known retired dye chemist in the United States), was available, Rubner approached me with the question "What are you doing anyhow?" After explaining my work on the nitrogen minimum, he at once became interested and soon I gained his confidence on a lasting basis. Today the biological value of proteins is an established fact. However, this value is no longer determined by nitrogen-balance experiments but by weight control of the growing animal, living on a special diet deficient in one essential amino acid (1).

In 1913 I became acquainted with Graham Lusk in Berlin. Through his widely read textbook my work on the biological value of proteins became more and more known although, personally, I never returned to these studies since I was dissatisfied with the nitrogen-content determination as a true measure for the protein minimum. In those days the nutrition scientists still claimed that animals were not capable of assimilating ammonia or inorganic nitrogen compounds. F. Knoop's observation that keto acids can be converted into optically active acetylated amino acids in normal metabolism was not yet widely known. The general belief was that only plants and micro-organisms were capable of this nitrogen assimilation of nitrogen. From Knoop's work it became clear to me that the biological value of proteins depended less on their nitrogen content than on the carbon skeleton of the in-

volved amino acids. Unfortunately, at that time there were no methods to determine amino acids quantitatively in hydrolysates. Colorimetric and spectrophotometric methods did not exist nor had paper chromatography been rediscovered. Hundreds of grams of protein were necessary to obtain even a fraction of the amino acids from the hydrolysates in a crystalline state. I confess that I did not think it possible that within only a few decades all these experimental difficulties could be overcome.

However, I used the above carbohydrate diet free of nitrogen, producing the true nitrogen minimum, in order to solve other questions. (a) How much nitrogen will be excreted when the entire need of calories is supplied by fat? In this case it was found that as much nitrogen is excreted as in complete hunger. The quantities of nitrogen excreted are the same whether the necessary calories are supplied by the body's own fat or externally by a fat diet. (b) What percentage of the calories provided by carbohydrates can be substituted by fat without affecting the minimal excretion of nitrogen? The answer is that nearly 95 per cent of the calories can be replaced. This means that 30 to 50 gm. of glucose fed daily are necessary to keep the lowest level of nitrogen in the urine. Without this quantity of glucose 4 or 5 gm. of nitrogen are excreted instead of 2 gm. According to Lusk an increase of 3 gm. nitrogen corresponds to 12 gm. glucose only, which in the best case could originate by neoglucogenesis. Here was a discrepancy demanding further investigation. (c) What kinds of nitrogen compounds occur in the urine in each kind of minimum? It could be shown that an increase of nitrogen in the case of a deficiency of carbohydrate is attributable to urea only. The excretion of creatinine is the same in both cases. Incidentally, 30 years later, I repeated this same nitrogen-minimum self-experiment for other reasons. Although I had gained considerable weight in the meantime, I excreted as much creatinine as previously. (d) In the case of nitrogen minimum on a fat diet, a co-worker and I excreted acetone. Is it possible to use this relatively simple experimental arrangement in order to learn more about the conditions under which acetone appears and reappears in the urine after intake of sugar? In our self-experiments we used butter fat mostly because we found it difficult to eat other kinds of fat, free from nitrogen, as the only diet. According to the literature, butyric acid was expected to give more acetone per mole than the long chain acids. Therefore, later on in Leipzig, we fed a pig, not yet fully grown, freshly distilled purest oleic acid with a stomach tube. We chose this acid which is easily resorbable in order to eliminate the possibility of sugar formation through the glycerol component of the fat. We decided on pigs because they, like dogs, do not have a tendency to excrete ketone compounds. Finally, a young pig was selected because it utilizes protein more economically than a fully grown animal. The ketogenic/antiketogenic ratio was found to be far beyond the usual range, but we could not obtain a constant value (2). Unfortunately, no unambiguous experimental arrangement existed which could have served to study the formation and excretion of acetone or ketone compounds *in vivo*. Otherwise, I would have continued these studies which

centered around a problem of interest even today. We know now, however, that ketone compounds are formed in the liver from the active acetic acid, the other intermediate of fatty acid metabolism.

During the second half of the first World War the study of nutritional problems in Germany became of major importance in view of the food blockade by the Allies. Food of vegetable origin had to be consumed to a greater extent, particularly potatoes. Rubner had developed an improved method to determine vegetable cell membranes which could be applied to determination of roughage in faeces (3). Today these determinations are made more easily with the N^{15} isotope.

Once more we considered the old question, to what extent should wheat be milled. No new aspects came to light which went beyond McCollum's book, *The Newer Knowledge of Nutrition*, published in 1918. The significance of vitamins was not fully understood in spite of the treatise on vitamins by C. Funk which appeared in Germany in 1914. The bread question arose once more during the German economic depression in 1930, which forced the government to curtail the import of wheat. The German government approached me for medical support of its propaganda for more complete milling of wheat. I declined to share the responsibility for measures dictated by economic and political reasons rather than by medical aspects (4). Research should never be exploited for political propaganda.

Returning to the nitrogen-minimum experiments with fat and carbohydrate, we found the creatinine excretion unaffected by either diet regardless of whether the metabolism measured was taken at rest or during exercise. Therefore the origin of creatinine was of particular interest. Creatine was considered a "food" in those days according to O. Folin. I thought of tagging its precursors by introducing a phenyl group in a manner similar to that used by Knoop with fatty acids. For some reason this work remained unfinished and nothing was ever published about it. Our records were lost in World War II. However, I explored the creatine problem in another direction later (5). Knoop has pointed out that the degradation of arginine to guanidino-acetic acid occurs according to his rules on the degradation of amino and fatty acids in the animal metabolism. The methylation of the guanidino-acetic acid to creatine has been observed by M. Jaffé, although on a small scale only; whether this happens *in vivo* remained uncertain. Enzymes often react very specifically; urease does not split methyl urea nor methyl arginine, as K. Felix could show with our preparations. Therefore, I thought that the introduction of the methyl group into arginine might lead to an increase in the creatinine excretion in the urine and if so, would support Knoop's idea. During the following decade I tried to prove this hypothesis, but neither starving rabbits nor patients afflicted with dystrophia muscularis progressiva, type Duchenne, ever excreted more creatine in the urine when fed with DL-methyl arginine or methyl-guanidino-butyric or -caproic acids. It is well known that creatine originates by a synthesis *in vivo* as R. Schönheimer could prove later on by using radioactive tracer elements. Furthermore, knowledge

of the transmethylation process lends support to this. Even today the question is not completely solved: why is creatine, or a functional derivative of it, converted to the same quantity of creatinine and why does the quantity depend on the weight of the muscles and not on the work load imposed on the muscle? I spent much time and effort to answer these questions because it was my belief that amino acids remaining organically combined in the protoplasm may undergo reactions in another manner and with other enzymes than would the so-called free amino acids originating from food proteins by hydrolysis. The latter always yield urea rapidly while the nitrogen from the former remains attached to its carbon atom. Rather early I formed the opinion that chemical reactions in the metabolic process may depend on the very complex and specific structures of the components, and one must realize that one deals with topochemical reactions at interfaces and not with ordinary chemical reactions in solutions governed by the law of mass action (6).

Free terminal groups in protein.—Another problem of great personal interest to me became the determination of the free terminal groups of proteins (7). We first tried to render them stable to hydrolysis. Casein was methylated and its hydrolysate used in feeding experiments. All natural unchanged amino acids disappeared in the metabolism. However, it was hoped that the betaines derived from methylated amino acids in the protein could be isolated from the urine. At that time we still believed, with Ackermann, that betaines would pass unchanged through the animal digestive system. Today we know that this is not always the case. To a small degree they can serve as methyl donors. We succeeded in isolating from urine only a lysine "betainized" on its ϵ -amino group.

Since the guanidino group withstands hydrolysis by concentrated acids, E. Schütte guanidated proteins, once more using an improved technique. Another approach was followed by my co-worker F. Bettzieche who applied the Grignard reaction to amino acids and peptides. Thereby the free carboxyl group was converted to a tertiary alcohol and was to be identified in this manner. In certain cases the resulting tertiary alcohols, however, did not stand the conditions of acid hydrolysis but decomposed to aldehydes, ketones, and ketonic acids, thus complicating beyond hope the quantitative isolation. The time was not ripe for a solution of problems of this type. Neither did we deal with proteins of uniform quality nor was paper chromatography rediscovered. Today, fortunately, one need not resort to animal metabolism to answer these questions.

Incidentally, Rudolf Schönheimer, an M.D. like myself, completed his chemical training at our laboratory in Leipzig, in 1927, thanks to funds obtained through the Rockefeller Foundation, an arrangement I will describe later. Schönheimer's later classical experiments in the United States with tracer elements in rats made him well-known and laid the foundation for the modern isotope technique in biochemical studies. I deeply regretted the premature death of this promising scientist.

My own experience had taught me how valuable an additional chemical

training can become for a medical man. It gave me great satisfaction to be able to organize with the support of the Rockefeller Foundation, a two year chemical training course for medical doctors in my laboratory in Leipzig. This organization had generously granted to my institute funds set aside for medical education and maintained them for a period of seven years. A good number of young capable medical doctors took this course to the great benefit of their future careers. The first year of this course was devoted to analytical and synthetic laboratory work; during the second year they participated in the research program of the institute either individually or by forming teams.

In 1928 one of my closest associates, J. Kapfhammer, accepted a professorship in Freiburg and soon afterwards another associate B. Flaschenträger, now living in Alexandria, Egypt, became professor in Zürich, Switzerland. Erich Strack joined the laboratory and now heads the Institute in Leipzig.

He once more studied the problems of exclusive nourishment (8). Feeding techniques were improved, and the dogs received flexible probes—paraffined Nelaton catheters (those made of inert plastic did not yet exist). The connecting tube left the body at the neck and usually healed in without irritation. The flexible probe was pushed forward through an esophageal fistula opening to the duodenum, as in modern heart catheters through the neck artery close to the heart. An automatic device guaranteed a well-regulated uninterrupted infusion (solution or emulsion). Thus, we could eliminate volition in eating and irregularities in the metabolism as a result of shock. The animal was free to drink as much water as it pleased. In this manner we fed internally great quantities of oleic acid or glycerol, thus providing sufficient calories. Intravenously, for instance, we gave, with and without insulin, triple doses of glucose without producing glycosuria. In other experiments distilled water was infused until great edemata were formed whereupon we turned the glass cock and changed the infusions to a concentrated Ringer solution; the kidneys usually began to refraction at once. After several hours the edemata disappeared, and the dog resumed its normal shape.

Ultimately, the metabolism cage was converted into a respiratory apparatus. During the automatic infusion, energy metabolism by indirect calorimetry, could also be measured. This permitted us to determine the fat formation in the case of excessive glucose feeding. If dogs received large quantities of pure glucose continuously for one week, they died. Autopsy revealed enlarged livers containing more than 20 per cent glycogen and hemorrhages in different areas. Today I explain this pathological picture as having been caused by an acute deficiency of biocatalysts, e.g., vitamins of the B group, or perhaps, by rapid depletion of essential elements without replenishment. These results, however, were insufficiently reproducible since the animals were taken from the city pounds and possessed different reserves of these substances.

Naturally, experiments of this nature are very expensive. Even with the automatic feeding device the animal had to be watched day and night. Absolutely pure substances were essential for the infusion experiments. Im-

purities, hardly detectable analytically (isomaltose or glucose-decomposition products originating during sterilization), can have very disturbing effects. Usually two physicians, two technical assistants, and one chemist supported by the institute work shop teamed up in one animal experiment.

With the aid of the Rockefeller Foundation such arrangements were possible until about 1933. After that time my younger associates became (often against their own will) engaged in political activities and some were drafted into the military service. Under these conditions their interest in research abated. Finally not only the apparatus but unfortunately, many of the records were lost as a result of enemy bombing in 1943. Since many of the experiments were still incomplete, only little was published in the "Berichte der Sächsischen Akademie der Wissenschaften" (9). This work should be resumed some day.

Our approach was not the conventional one. Instead of eliminating essential elements, vitamins or biocatalysts, from the diet we first produced the deficiency, e.g., avitaminosis, by feeding an over-supply of nutritive substances known to cause depletion of these biocatalysts. This was done by glucose, which under natural conditions enters the metabolism irregularly and shockwise. Nature somehow makes provision to absorb these shocks. Experiments of this kind with other metabolites also could, in my opinion, be continued with a good chance for interesting results. Despite the wholehearted efforts of all concerned, these studies had to remain incomplete as a result of conditions beyond our control. I should like once more to express my thanks to the Rockefeller Foundation.

Fat metabolism.—In connection with our work on creatinine we had prepared ample quantities of ϵ -amino caproic acid (not readily available at that time) and had substituted the amino group with benzenesulfonyl and methyl groups. In feeding these compounds, which possess the solubility of a fatty acid the β -oxidation stopped at the butyric acid stage. Similarly substituted ω -amino acids with an odd number of carbon atoms in the chain were oxidized only to the propionic acid stage. This configuration prevents β -oxidation of an additional two carbon atoms, as in the case of phenyl substitution where the degradation proceeds to the phenyl acetic acid and benzoic acid respectively. If the α -amino group be substituted, the degradation begins at the other end. From the substituted α -amino lauric acid Flaschenträger obtained adipic acid. If the carboxyl group be converted into an amide, degradation likewise begins at the other end. Sebacamic acid is easily oxidized, in comparison to sebacic acid.

Around 1920 I became engaged, together with my co-workers H. Schotte and B. Flaschenträger in the metabolism of fat, work which Flaschenträger continued later in Zürich together with K. Bernhard (now in Basel) (10). In the meantime P. E. Verkade had demonstrated the ω -oxidation of specific fatty acids of medium chain lengths as a normal occurrence. I returned to this problem in 1937. After 1933 the German economy was geared to become more independent of import. Participation of the academic research labo-

ratories in Germany's second four-year plan became mandatory. Rather than being forced to accept a government research order, I suggested an investigation of the biological value of synthetic fat as a food component which then became available for the first time from the hydrogenation of coal. At the start I realized that this was a formidable program because synthetic fat was really a new proposition to the biochemist, and many aspects had to be explored. In 1940 Dr. G. Weitzel joined in this work which was continued without interruption until the end of the war, sometimes under most difficult conditions. The very first feeding experiments with dogs indicated that the greatest part of the synthetic fat is absorbable and therefore, a priori, suitable to supply the necessary calories. However, it is not oxidized completely (11); unusual residues appear in the urine, particularly aliphatic dicarboxylic acids. Only a small part of these could be isolated and identified. We found the series of normal saturated di-acids from C_4 to C_{10} . However the bulk was an oily mixture which resisted further identification. Most likely it consisted of different acids with branched carbon chains since the starting material, synthetic paraffin, contains both straight and branched chain hydrocarbons. Therefore, we prepared synthetically a great number of branched aliphatic dicarboxylic acids in order to study their physical, chemical, and biological behavior. K. Bernhard investigated the series of alkylated malonic acids, and we examined succinic, glutaric, and adipic acids. In summarizing this work one simply cannot say that all branched chained fatty acids are toxic and cannot be resorbed; that is not true. It always depends on the type, the number, and the location of the side chains. In most cases one or several methyl groups do not interfere. As Verkade already had proved, the length of the chain is also of importance.

While synthetic fat lost its importance after the war as a result of the availability of natural fats, our work with it had led us to a number of new fundamental problems which formed the basis of a new research program. Since this work has been reviewed recently by G. Weitzel and myself and ample literature citations have been given (11, 12), only some of the more significant results of our investigations may be mentioned here. G. Weitzel, A. Fretzdorff, and S. Heller (13) further developed and perfected an automatic precision method of studying monomolecular (Langmuir) films. This method permits the observation of fine differences in chemical structure and shape, as well as measurements of the spatial requirements of the molecule, all within a few minutes with excellently reproducible results. In possession of this reliable and highly sensitive method we were able to study, systematically, long chain fatty acids in model experiments. In due course we evaluated branched chain fatty acids in animal metabolism, and we were able to interpret the existence of such branched chain acids in tubercle bacilli. We believed that the occurrence of branched chain fatty acids in nature was relatively rare until we found them abundantly in the wax of the preen glands of birds. The biological significance of the peculiar chemical composition of this wax became clear to us. The special physiological role of fatty acids of

medium chain length (C_8 to C_{12} acids) was again recognized. Their application in the therapy of noninfectious skin diseases, e.g., psoriasis, acne vulgaris, etc., was suggested as well as their potential importance in the therapy of tuberculosis was pointed out. In general, we made some progress in correlating chemical constitution with specific biological functions of fatty substances. May I say, in summarizing, that our work on synthetic fat has added a new chapter to the heretofore meager knowledge of lipide metabolism.

Very recently we became interested in two new problems. Weitzel began to study complex zinc compounds and their biological behavior in tissue, and I began to devote some time to the study of certain occupational diseases like silicosis. Our institute is well suited for this type of work in contrast to some university laboratories, since we can attack such questions with teams of chemists, physicists, biochemists, and physiologists.

CONCLUDING REMARKS

A few more personal remarks may be added at this point. How far have inherited abilities and acquired knowledge contributed to my professional career, I do not know. Did I derive professional satisfaction and happiness from my work? I am certain of that and yet, I cannot prescribe any formula on how I achieved it. Perhaps I can express myself more clearly in the words of the great German poet, Goethe:

*Eines schickt sich nicht für alle.
Sehe jeder, wie er's treibe,
Sehe jeder, wo er bleibe,
Und wer steht, dass er nicht falle.*

I went through life fairly well on my own. I never married and yet, my associates have never thought of me as the lone wolf type because I always found happiness in working with and for others and for science; the financial rewards meant little to me. I always got what I required for my simple life, although my parents could not provide much for me. I lost my savings twice through no fault of my own: in 1920, by the inflation of the German currency and again, in 1945, by Germany's collapse and my subsequent evacuation. I no longer cling to worldly possessions which the troubled times of my generation have proven to be of doubtful value. Only spiritual values last, at least during one's lifetime. Otherwise, I could not have started my career again and achieved success at an age when others retire. At the age of 63, by evacuation from the Eastern Zone, I even lost the claim to my pension as a government employee. However, I am greatly indebted to the Max Planck Society for all my later achievements. Without its aid the new research center in Göttingen, where 100 people work, study, teach, and investigate would not be in existence today. I must confess that I never knew an eight hour day in the laboratory nor demanded it from my collaborators. The "exempla docent" always sufficed. It is always worthwhile to follow a scientific problem intensively, with the best available techniques and as thoroughly as possible. The result may be something unlooked for, but in any case a

collection of experimental data will be useful sooner or later. As Richard Willstätter put it, this is the only reasonable thing a research worker can do. I fully agree with him, and this attitude towards my profession has never left me during my entire career. Of course, one must recognize in time when a specific field has been exhausted and for the time being appears unpromising, even with a new approach. My teacher, Rubner, was a physiologist; he became a hygienist and then returned to physiology. He told me once: "When young, one has to look for a broad and thorough training. One never knows which tasks and duties one may be expected to perform." Therefore, he understood why I at the age of 30, a time when others usually have a family of their own, packed up and went to college again to study chemistry.

I consider the series of lectures which every German professor is required to give, as another valuable contribution to a sound training. It compels him every year to think through his subject matter again and keep abreast of the literature. When I prepared my lectures, new ideas often came to me while thinking over, simplifying, or elucidating the subject to be presented. Unsuccessful lecture experiments leading to a search for the cause or failure also proved helpful. There are many examples of this in the history of chemistry.

Fifty years ago, there existed in Germany, the mother country of physiological chemistry, only two professorships for this subject,—one in Tübingen and one in Strassburg, both founded by Hoppe-Seyler. In all other 20 German universities physiological chemistry was taught by a physiologist with an M.D. degree. Exceptions were Albrecht Kossel (Heidelberg) and Emil Abderhalden (Halle) who occupied physiological chairs in spite of their predominantly chemical education. There has been no basic change since those days. Of course, every medical faculty has its own biochemical professorship. D. Ackermann, F. Knoop, and the Gesellschaft für Physiologische Chemie pleaded for it again and again. Most of these biochemical institutes have very limited means; the number of medical students has multiplied during the past 50 years but not that of the teaching staff. New, adequate, institutes arose only in the laboratories of the major hospitals. Their superior equipment often leaves the university laboratories far behind. They pursue mainly "applied research." The trend of this development is attributable to the social legislation in Germany. Under these conditions in Germany the supply of young teachers in physiological chemistry remains scarce.

If one has to look for the means of doing research, a certain flexibility is necessary. I adjusted my research objectives to external conditions. I began with nutritional research, because I was associated with Rubner, and, in its further development, I came to physiological chemistry because this subject interested me greatly. I obtained the professorship at Leipzig. I turned to fat physiology because in this field I could escape political pressure, and, finally turned to pneumoconioses because I wished to leave the subjects dealt with previously to my collaborators, and because I could obtain industrial support for the institute by working on occupational diseases. At the beginning of my career as laboratory director with full responsibility I did not want to

accept any contribution from industry, because I did not want to sell my freedom. However, when governmental support ended and it no longer observed a neutral attitude towards research, I had to change my attitude and make some concessions. This was accomplished best by asking for the support of development work on a larger comprehensive program and convincing the sponsor that his money would be spent wisely.

I consider myself fortunate that I could work the major part of my life in academic institutions and not in pure research laboratories. Close contact with the younger generation is vital for progressive thinking. I do not deny that there should be institutes of pure research; they have functions and tasks of their own. However, such institutes should not serve a limited narrow field. Their name should indicate a general comprehensive purpose, and they should be built in university towns; in close proximity to the academic institutes, even in these days of the automobile. A neighborly arrangement of this sort will further acquaintanceships, encourage exchange of thought between the members of the younger generation, and prevent a stagnation of the research institute in its limited field. Having been active in directorial positions in both universities and institutes of pure research, I can speak from my own experience.

Fifty years ago the main goal of biochemistry was the discovery of all components of the living cell and elucidation of their chemical structure, as it was in Liebig's day. Its attitude was static. I may mention the work of A. Kossel and F. Kutscher on the basic amino acids and of D. Ackermann, one of their pupils, on the basic tissue extracts, of Hans Thierfelder who investigated the lipides and also Emil Fischer, A. Windaus, H. Wieland, R. Willstätter and others. The last named were professors of chemistry; their pupils again are the heads of chemical laboratories. Because of their many collaborators, their work was much more fertile than ours.

This "static" biochemistry laid the foundation for the present "dynamic" phase of metabolism research. Franz Hofmeister (Prague and Strasbourg), Gustav Emden and Karl Spiro (Strasbourg, Frankfurt, and Basel), Franz Knoop (Freiburg and Tübingen) were the earliest and most successful workers in this field in Germany. I have discussed already how they carried out their investigations on the whole animal. Even today I consider this "old-fashioned" method very necessary. It reveals what the organism as a whole can accomplish. Only afterwards comes the elucidation of the process or, preferably, of the detailed reaction sequences, which in most cases are quite complicated. Such studies require a thorough knowledge of the individual enzymes involved and their kinetics. Of Germans I mention here especially H. Wieland and F. Lynen, O. Meyerhof and K. Lohmann, Carl Neuberg, and especially Otto Warburg and his school, from Meyerhof and H. A. Krebs to Th. Bücher. Together with A. Tiselius, Otto Warburg has been instrumental in developing new techniques. When Warburg started, yeast zymase was considered a single enzyme which effected the alcoholic fermentation of sugar. Mainly on the basis of his work zymase today has been divided into

11 enzymes and the fermentation process can be subdivided accordingly into many well known individual steps. This new branch of biochemistry has already become a field by itself. Chemists and physical chemists do team up, because the nature of the work requires thorough training in both fields as well as experimental skill for successful and reliable work.

Nevertheless, I should like to see a place for the physician too in the biochemistry of the future. We must not forget that many questions had their origin at the hospital bed. Today such questions can be solved much more exactly than formerly by using radioactive tracer techniques in studying animal metabolism. Physiological chemistry has made a tremendous advance during the past two decades by studies with isotopes; unfortunately, this technique is still in its infancy in Germany. At its founding I already had made the Medizinische Forschungsanstalt in Göttingen the center for the import of isotopes from other countries and the control of their use.

I have always been fortunate in finding congenial collaborators. Some who remained in our branch I have named: Kapfhammer, Flaschenträger, Schönheimer, Strack, Schütte, Weitzel. Space limitations prevent me from naming those who were killed during the two world wars or who have returned to practical medicine. In the case of the latter, was the seed deposited in vain? I do not think so. Clear thinking and constructive criticism are beneficial for every man and every branch. Therefore, many clinicians have profited from their biochemical training, although this fact is often not too readily apparent. Perhaps I have been too altruistic and too little of an egotist, as seen from the standpoint of my specialty of physiological chemistry, and have turned the principle of the Prussian kings "suum cuique" too much into "meum cuique" in line with my nature and my upbringing,

*"Und doch, wär' mir die Wahl gegeben,
Ich führte noch einmal daselbe Leben;
Und sollt' ich noch einmal die Tage beginnen,
Ich würde denselben Faden spinnen."*

(Th. Fontane)

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BIOLOGICAL OXIDATIONS^{1,2,3}

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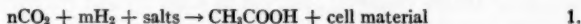
The evolutionary process has, by its emphasis on efficiency of utilization in the face of ever-increasing disregard for environment, imposed on the cell a number of successively more involved metabolic mechanisms requisite for survival. Where the strictly anaerobic microorganism may sacrifice pyruvate lavishly as diffusible alcohol in the course of electron disposal, the mammalian cell persists with its thorough dissection of organic structures until only the skeletons, CO₂ and H₂O, remain. These oxidation-reduction reactions are coupled to mechanisms which permit the accumulation of free energy released, in a form useful in biosynthetic processes.

The concept of the importance of "group potential" (1) in biosynthesis appears to be a general one at all levels of life. Thus reshuffling of functional molecular groups and structures can occur from one pool of substances to others without significant change in free-energy content. Superimposed on these horizontal redistributions, however, is the vertical distribution of such systems on the EMF scale of the evolutionary process.

In this review we shall consider recent advances in knowledge for each of the successively more electropositive coupling points in the biological EMF series in relation to the intermediary oxidative systems for which those points act as electron-accepting coenzymes.

HYDROGENASE AND FORMIC DEHYDROGENASE

In rare instances, biosynthesis of cell material can occur in the presence of H₂ as the sole available oxidizable material. One such instance is the case of *Clostridium acetivum* (2) which carries out the over-all process



The potential drop from the E₀' levels of the hydrogenase reaction, H₂ → 2H⁺ + 2e, to the reaction, DPN + 2H⁺ + 2e ⇌ DPNH⁺ + H⁺, is 0.10

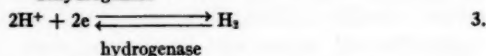
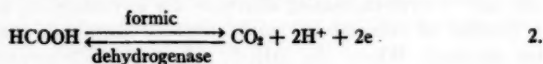
¹ The survey of the literature pertaining to this review was completed in October, 1953.

² The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DNP for dinitrophenol; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavin-adenine-dinucleotide; INH for isonicotinic acid hydrazide; LAD for liver alcohol dehydrogenase; LTPP for lipothiamide pyrophosphate; PCMB for *p*-chloromercuribenzoate; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); TPP for thiaminepyrophosphate; YAD for yeast alcohol dehydrogenase.

³ The authors wish to express their appreciation to Drs. E. R. Stadtman, T. C. Stadtman, B. L. Horecker, and H. M. Kalckar for their many helpful suggestions during preparation of this manuscript.

volts and only about -2300 calories of free energy per electron can be produced. It is clear, therefore, that more electropositive end electron acceptor systems must be operative if we accept the general premise that biosynthesis requires the net formation of bonds with heats of hydrolysis of the order of 12,000 calories per mole.

The free energy change in the formic hydrogenlyase system:



is essentially zero (3). Therefore, the growth of organisms such as *C. acetium* must be geared to a system in which, starting with formate or a formyl derivative, fermentations take place that can result in the eventual accumulation of acetate, CH_4 , etc., as electron-terminating compounds. The details of such intermediary reactions are completely unknown.

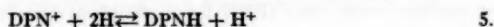
For reasons of experimental convenience, the mechanism by which H_2 gas is converted to hydrogen ions and electrons has been studied mainly in higher organisms where H_2 can replace organic electron donors (e.g., ethanol butyrate).

Although previous studies have not implicated DPN or TPN² as components of the hydrogenase system, more recent work on *Clostridium kluyveri* (4) indicates a direct coupling with these nucleotides. This hydrogenase system requires, in addition to a DPN-linked acceptor system, a cation probably Fe^{++} , as well as an unidentified heat stable factor (not replaceable by TPP²). Whether this factor is identical with the hydrogenlyase yeast factor of Lichstein & Boyd (5) has not been determined. The stimulation by TPP and pyruvate (or diacetyl) observed by Gest (6) and the pyridine nucleotide coupling mentioned above in reaction 4 suggest the possibility that the fully integrated formic hydrogenlyase system may combine DPN or TPN-linked oxidation-reduction reactions with TPP-linked "active-acetaldehyde" transfers (see section on α -keto acids) such as are suggested by Gest's results.

Various energetically feasible schemes can be put forth to describe such a situation, but are not presented here since they involve the postulation of undemonstrated reaction sequences.

PYRIDINE NUCLEOTIDES

Thermodynamic constants.—During the past year two papers have appeared which establish a corrected value for the E_0' of the reaction



From the equilibrium constant of the isopropanol-DPN-acetone reaction catalyzed by alcohol dehydrogenase, combined with reliable free-energy data for isopropanol and acetone, Burton & Wilson (7) have calculated the

E_0' value for the DPN/DPNH couplet to be -0.320 v. at pH 7. Although the calculated value of Borsook (8), -0.28 , was earlier confirmed in direct titration experiments by Rodkey & Ball (9), Rodkey (10) has more recently redetermined this value, and, employing careful corrections for destruction of DPN during the titration process, has obtained a value essentially identical with that of Burton & Wilson.

Accepting this value of -0.320 v., the E_0' for the TPN/TPNH² couplet becomes -0.324 volt, on the basis of the equilibrium constants determined for the glutamic acid dehydrogenase-catalyzed equilibria with DPN and TPN as coenzymes (11).

The new value for the DPN/DPNH couplet also requires a quantitative change in the value calculated by Oesper (12) for the free-energy of hydrolysis of ATP. This value now becomes $+8,640$ calories, using Oesper's equations and assumptions without modification.

Chemistry and catalytic function of pyridine nucleotides.—Essentially all biosynthetic energy production is dominated by reactions linked through pyridine nucleotide mediators. In the case of pyruvate oxidation, for example, 13 of the 15 ATP² molecules synthesized from ADP² appear to be derived from such reactions. Upon inspection of this class of oxidation-reduction reactions, it becomes evident that they fall into two major groups, those involving only "simple" electron donor-acceptor processes, and those which also incorporate "high-energy" group transfer processes. In the latter group, recent experience begins to suggest that, in general, two coenzyme functions are required. Thus, for example, both pyruvate and α -ketoglutarate oxidation involve lipoic acid or its derivatives in addition to pyridine nucleotide, and in the case of triose-phosphate oxidation, enzyme sulfhydryl appears to be essential. The case of glyoxylase at present constitutes an exception to this generality, since a thioester bond is formed (lactoyl-glutathione) without the addition of coenzymes other than glutathione (13).

In the following section, we shall consider, first, some of the recent findings on pyridine nucleotide chemistry and second, new observations on both old and new enzymes in the two classes of dehydrogenases mentioned above.

Several important advances have been made during the past year toward an understanding of the details of pyridine nucleotide reduction and the stereochemical specificity of dehydrogenations involving this class of coenzymes. The long-accepted assumption that addition of substrate hydrogen atoms occurs at positions 2 or 6 of DPN during dehydrogenase action has been critically examined (14). The unexpected conclusion derived from these studies is that such addition takes place at neither of these positions but rather on position 4 of the pyridine ring. Thus when DPN chemically reduced in D₂O, was degraded enzymatically and chemically to 1-methyl-2-pyridone-3-carboxamide and 1-methyl-3-carboxamide-6-pyridone, the two derivatives contained equal amounts of deuterium. This observation is best explained by the scheme shown in Figure 1 in which the 4 position is unaffected by the oxidation procedure.

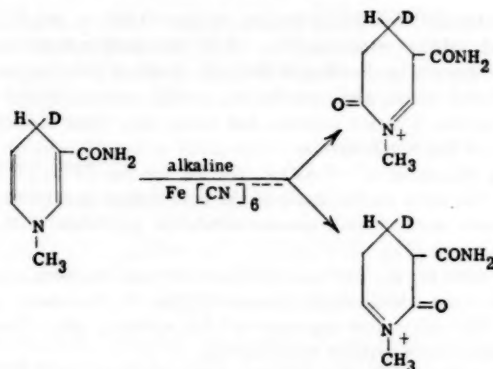
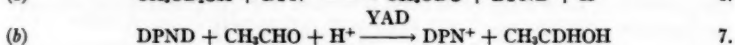
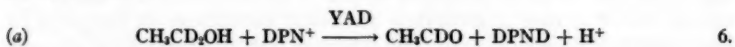


FIG. 1. Oxidation of deuterio-DPN with alkaline ferricyanide.

Westheimer & Vennesland and their colleagues have continued their studies on the stereospecificity of DPN-linked dehydrogenase reactions. In their previous experiments (15, 16) they had shown that yeast alcohol dehydrogenase (YAD) catalyzed the transfer of deuterium from deuterioethanol to DPN and that the resulting DPND contained one atom of deuterium per mole. When this DPND was reacted with nonlabeled acetaldehyde under catalysis by the same enzyme, the resulting DPN was devoid of isotope. Thus,



These results suggested that the hydrogen on only one side of the plane of the pyridine ring of DPN was enzymatically involved in the reaction studied.

In the more recent work by this group (17), the monodeuteroethanol produced in reaction 7 above has been incubated with DPN and yeast alcohol dehydrogenase, yielding acetaldehyde containing no deuterium. Thus, it appears that only one enantiomorph of CH_3CHDOH reacts and that YAD³ is stereospecific for both ethanol and DPN. It was further shown that DPND, prepared enzymatically as described above, transferred its deuterium to pyruvate in the presence of lactic dehydrogenase (18), yielding lactic acid containing one mole of deuterium per mole. Thus, this enzyme appears to have the same stereospecificity for DPN as does YAD. These investigators present a schematic model structure for the postulated enzyme complex shown in Figure 2. They state: "... steric specificity, together with the demonstrated 'direct' hydrogen transfer, can be explained most readily in terms of a mechanism which involves the binding of both substrate and co-

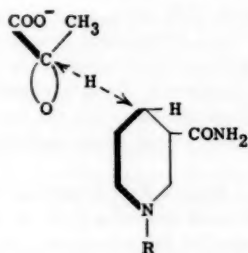


FIG. 2. Three-dimensional orientation of lactate and DPN during enzymatic oxidation.

enzyme in adjacent positions at the active site of the protein, with such three-dimensional orientation that hydrogen can be transferred from (or toward) one side only of the pyridine ring to (or from) the carbonyl carbon atom of pyruvate in only one particular steric position (see Fig. 2). (The diagram is not intended to represent absolute configuration or to imply a choice between any particular one of the possible spatial arrangements of the molecules.)"

A similar ternary complex has been indicated by the studies of Kaplan & Ciotti (19) in the case of yeast LAD², DPN, and hydroxylamine. The latter, a potent inhibitor of the enzyme, appears to complex with the specific protein and DPN, the resulting inhibition of ethanol oxidation being greater following preincubation with the inhibitor. Lowering the pH of the final reaction mixture from 10 to 7 decreases the degree of inhibition of LAD but not of YAD.

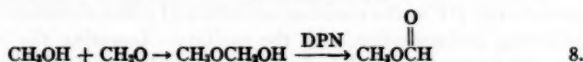
In continued studies on DPN analogues, Zatman *et al.* (20) have employed the nicotinamide exchange process observed with spleen diphosphopyridine nucleotidase (21) (see review by Kalckar) to prepare the INH² analogue of DPN. Observing that different species varied in respect to the inhibitory activity of INH on various tissue diphosphopyridine nucleotidases (22), they subsequently found that, aside from the *Neurospora* enzyme, the "INH insensitive" enzymes led to formation of a product of yellow color in alkali (20). Subsequent analysis led them to conclude that this was the INH analogue of DPN.

Burton & Kaplan, during their study of glycerol dehydrogenase from *Aerobacter aerogenes* (23) observed an interesting nonenzymatic reaction of dihydroxyacetone and DPN. N-substituted nicotinamide and α,β hydroxy-keto compounds were found to react in alkaline medium to give products with an ultraviolet absorption spectrum identical with that of reduced pyridine nucleotides. In the case of DPN and dihydroxyacetone, the product was not identical with DPNH since it failed to replace this compound in enzymatic reactions.

Stafford & Vennesland (24) have studied the alcohol dehydrogenase activity of wheat germ. Like crystallized liver alcohol dehydrogenase (25), extracts of wheat germ will oxidize ethanol in the presence of TPN at somewhat less than one one-hundredth the rate observed with DPN. Since the relative reactivity of various alcohols with the two pyridine nucleotides was different, it is possible that wheat germ contains more than one alcohol dehydrogenase.

An unusual action of alcohol dehydrogenase has been observed during investigations initially directed toward a study of ethanol inhibition of methanol oxidation by liver alcohol dehydrogenase. In attempting to assess the "aldehyde mutase" activity of their alcohol dehydrogenase preparations, Kendal & Ramanathan (26) observed that in the presence of methanol the disappearance of formaldehyde was accelerated without a corresponding increase in acid production, though the discrepancy decreased with time. It was subsequently observed that a volatile ester of formic acid (methyl formate) accumulated during the early stages of the reaction and subsequently underwent slow hydrolysis.

From the observation that 0.01 *M* iodoacetate did not inhibit the disappearance of formaldehyde in the presence of methanol (or ethanol) whereas, without the added alcohols, disappearance of formaldehyde was almost completely suppressed, it was concluded (26) that the enzyme responsible for ester formation was the alcohol dehydrogenase (known to be insensitive to iodoacetate at this concentration (27) and the reaction scheme proposed involved the preliminary formation of a hemiacetal, i.e.,



The nature of the linkage between the substrate, coenzyme, and yeast alcohol dehydrogenase molecules has been investigated by Barron & Levine (28). This enzyme, in contrast to the liver enzyme, was shown not to form a spectrophotometrically-observable DPN complex. Nevertheless, Barron & Levine demonstrated that the previous addition of ethanol or DPN to YAD protects the enzyme against the inhibitory effect of thiol reagents. They found also that such additions reduced the number of titratable sulfhydryl groups in the protein as much as 42 per cent. The results of these studies are compatible with the hypothesis proposed by Racker for the triosephosphate dehydrogenase mechanism discussed in a later section.

It is tempting to consider that such a mechanism for DPN-linked dehydrogenase action, involving sulfhydryl-DPN linkages, is a general one. Studies by Chance & Neilands (29), however, have demonstrated the formation of an enzyme-DPN complex in the case of lactic dehydrogenase, an enzyme which is PCMB³ insensitive. This binding, demonstrable spectroscopically when protein was present in stoichiometric excess of the DPN concentration, might represent the presence of an alternative type of DPN binding site, although the involvement of sulfhydryl groups not capable of

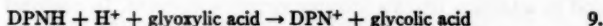
reaction with PCMB can certainly not be ruled out on the basis of present evidence. The existence of two forms of lactic dehydrogenase has been further investigated by Neilands (30) who has separated them electrophoretically. They differ apparently only in the number of free acid groups and are both enzymatically active. Another example of this polymorphism of enzymes has been observed by Krebs (31) with yeast D-glyceraldehyde-3-phosphate dehydrogenase. Krebs *et al.* (32) have also identified this enzyme with the crystalline "yeast protein 2" of Kunitz & McDonald (33), previously of unknown function.

Miller, Huggins & Arai (34) have recorded that propanediol phosphate, observed to occur normally in a number of tissues (35, 36), may be oxidized by crystalline myogen A, known to contain glycerophosphate dehydrogenase as well as aldolase and possibly a third component. The product of this oxidation is further oxidized by glyceraldehyde-3-phosphate dehydrogenase. From experiments with glycerophosphate the presence of isomerase was inferred. The products of the reactions were not identified. Nevertheless, a reaction mechanism is proposed postulating an aldehyde-phosphate as intermediate and an acyl phosphate (lactoyl phosphate) as final product. In view of the extensive investigations of the mechanism of glyceraldehyde phosphate dehydrogenase (see p. 25) the presence of an undissociated aldehyde phosphate in the reaction seems unlikely.

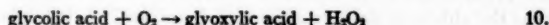
Glycerol dehydrogenases catalyzing the conversion of glycerol to dihydroxyacetone have been prepared from *A. aerogenes* (23) and *Escherichia coli* (37). On the other hand, the mycobacterium system studied by Hunter (38) appears to involve initial phosphorylation of the glycerol by ATP and metabolism through the usual glycolytic pathway.

Choline oxidase continues to be a source of numerous investigations (39 to 45) but relatively little is known of the characteristics of the primary process. Although this enzyme is apparently a specific alcohol dehydrogenase, no clear demonstration of a coenzyme-linked nature have appeared. The stimulation of methylene blue reduction by choline through the addition of DPN to a system free of betaine aldehyde dehydrogenase (41, 46) (known to be DPN-linked) leads us to include this enzyme as pyridine nucleotide linked.

An unusual electron carrier system of uncertain role in respiration has been observed in spinach leaves (47, 48). The system consists of glyoxylic acid reductase catalyzing the reaction



and glycolic acid oxidase (a flavin enzyme) catalyzing the reaction



Thus glyoxylic acid could function catalytically in the transfer of electrons from DPNH to O_2 . The two enzymes have been partially purified. The de-

hydrogenase functions with either DPNH or TPNH, although the rate of reaction with the latter is only about one-fifth that with the former. Glycolic acid oxidase has been shown to form adaptively in leaves of plants exposed to light (49). The adaptation appears to be attributable to the formation of glycolic acid during the photosynthetic cycle.

An enzyme of considerable interest in connection with its possible role in pyrimidine biosynthesis has been prepared from an unidentified anaerobic bacterium by Lieberman & Kornberg (50). This dehydrogenase catalyzes the reaction



The significance of this process is considered elsewhere in this volume.

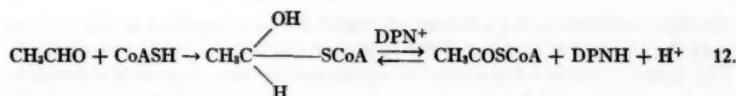
Chance has presented a summary (51) of the *in vivo* studies on DPN-linked reactions that he and his collaborators have undertaken in recent years. A "cycle" of reduction and reoxidation of the pyridine nucleotides, observed spectrophotometrically, was found to be initiated by the addition of glucose to starved yeast cells. The observed fluctuation without changes in respiration rate were attributed to the rapid reduction of DPN bound to triosephosphate dehydrogenase (estimated to be about 20 per cent of the total cellular DPN) with slower subsequent reactions in the DPN-linked system represented by this enzyme and α -glycerophosphate dehydrogenase. It is suggested that the steady-state of DPN in respiring cells is dominated by the DPN-linked dehydrogenases and not by the "respiratory enzymes" although the basis for this suggestion seems somewhat arbitrary since, in a steady state, the degree of reduction of any component in the chain of electron transport must be equally affected by both the slowest and fastest reaction rate involved.

A series of papers by Singer & Kearney (52) have appeared dealing with the oxidation of cysteine sulfinic acid by *Proteus vulgaris* and have suggested the involvement of a new pyridine nucleotide (Coenzyme III). The previous review in this series has already presented a summary of these observations.

ALDEHYDE DEHYDROGENASES

Two new aldehyde dehydrogenases have been reported. Seegmiller (53) has obtained an enzyme from yeast differing from that reported earlier by Black (54) in that it is TPN-specific whereas that of the latter functions equally well with either TPN or DPN. Both require divalent ions for activity and in addition Black's enzyme requires cysteine. In contrast to these enzymes as well as liver aldehyde dehydrogenase (55, 56), the enzyme obtained from *C. kluyveri* (57) requires CoA^2 for oxidation of acetaldehyde and the product is acetyl-CoA. Similarly, Pinchot & Racker have observed (58) that the aldehyde dehydrogenase of *E. coli* is CoA dependent.

Burton & Stadtman (57) have suggested that the mechanism of aldehyde oxidation by the *C. kluyveri* dehydrogenase may involve initial hemimercaptal formation, i.e.



An analogous hemimercaptal mechanism has been suggested (see p. 28) for the case of triosephosphate dehydrogenase.

GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

Glyceraldehyde phosphate dehydrogenase, aside from its position as catalyst in the primary oxidation reaction in glycolysis, possesses some unique qualities when compared with the other "active acyl"-producing dehydrogenases, systems also incorporating group transfer processes. Generally speaking, only one coenzyme (DPN) is known with certainty to participate in its activity.

Krimsky & Racker (59) have presented evidence that glutathione is persistently present in preparations of the dehydrogenase and is quite firmly bound, incapable of equilibration with free C^{14} -labeled glutathione.

It was initially observed by Harting (60) that triose phosphate dehydrogenase catalyzes the oxidation of acetaldehyde to acetyl phosphate. Subsequent studies by Harting & Velick (61) demonstrated that the enzyme also catalyzes (a) an exchange reaction between acetyl phosphate and P^{32} , (b) the arsenolysis of acetyl phosphate, or (c) a transacetylation reaction to glutathione or CoA. All these functions are consistent with the view that an acyl enzyme intermediate occurs in the normal reaction of the enzyme. Further characterization of these reactions included the acceleration of the phosphate exchange by cysteine and its dependence on the enzyme-bound DPN, although the exchange reaction is many times faster than the oxidation or reduction of DPN.

Although iodoacetate prevents the oxidation process promoted by the enzyme, it does not completely suppress the arsenolysis of acetyl phosphate, provided glutathione is added to the reaction mixture. On the basis of this observation Racker & Krimsky (62) have proposed that the glutathione observed in their dehydrogenase preparation functions as a prosthetic group of the enzyme. In view of the difficulty in assigning a function to glutathione in keeping with its ubiquitous character, it will be of interest to see if further investigation supports this hypothesis.

Velick *et al.* (63) have re-examined the binding of DPN by glyceraldehyde phosphate dehydrogenase. The DPN exchanges rapidly with P^{32} -labeled DPN, and in the enzyme, as isolated, 2 moles of DPN are bound per mole of enzyme. By ultracentrifugal separation and analysis of solutions of DPN and enzyme, they obtained a dissociation constant of 2×10^{-7} moles per liter for the enzyme-(DPN)₂ complex. On the other hand, enzyme from which the DPN had been removed by charcoal treatment bound a maximum of three DPN molecules per mole of enzyme on recombination. The dissociation constants, while clustering around the value given above, appeared to

decrease with increasing amounts of bound DPN, suggesting to the authors that the binding of the first DPN increased the affinity for the second, etc. The initial velocity of the reaction catalyzed by the enzyme is dependent only on the bound DPN. Estimates of the affinity of the enzyme for DPNH placed it at the same order of magnitude as that for DPN. Finally, the binding of DPN was independent of pH over the range of 7.0 to 8.3.

Velick (64) has also observed that the reversible inhibition of the enzyme by mercaptide forming reagents is complete when three —SH groups per mole of the rabbit muscle enzyme and two per mole of the yeast enzyme have reacted. By ultracentrifugal analysis of the enzyme with increasing concentrations of mercurial, it was found that the bound DPN was released in amounts which were a function of the amount of inhibitor, the process being complete when three moles of inhibitor had combined with the enzyme. Measurement of an increased absorption in the neighborhood of 340 to 360 $m\mu$ associated with the binding of DPN by the enzyme has been employed both by Velick (64) and by Racker & Krinsky (62) in their studies. Both laboratories observed that *p*-chloromercuribenzoate eliminated this spectral change as did iodoacetate (62), the former effect being reversed by cysteine (64). Velick has also observed that the absorption of the enzyme —(DPN)₂ complex at 340 $m\mu$ decreases with successive crystallizations. In part, this is associated with inactivation and can be restored with cysteine. Such reversibly inactivated enzyme still contains two DPN equivalents.

Racker & Krinsky have observed that the 360 $m\mu$ absorption also disappears upon addition of 1,3-diphosphoglyceric acid, acetylphosphate, or acetylglutathione. Segal & Boyer (65), studying the role of thiol groups in the activity of the enzyme, have reported that there are about 15 reducing groups in the enzyme as determined by amperometric titration with *o*-iodosobenzoate. Of these, 4 to 5 had reacted with iodoacetate when the enzyme was 80 to 90 per cent inactivated. The enzyme was protected against the action of iodoacetate by glyceraldehyde phosphate. This was also true for the enzyme from which the DPN had been removed by charcoal treatment. Analysis of the substrate protected enzyme treated with iodoacetate indicated that two of the SH groups that normally reacted with the iodoacetate were protected by the substrate. Experiments thus indicate that 2 to 3 thiol groups are involved in the interaction of enzyme and DPN and that two are also involved in interaction with the substrate. The experiments of Segal & Boyer suggest that the two involved in each case are not identical, but it is probably a little premature to present this as a conclusion, particularly since Racker (13) has observed that this "substrate" protection may be obtained with a wide variety of compounds.

Warburg & Christian (66) originally proposed that the reaction promoted by glyceraldehyde phosphate dehydrogenase proceeded through an initial nonenzymatic association of phosphate with the carbonyl groups of the substrate, subsequent oxidation producing 1,3-diphosphoglyceric acid. Meyerhof & Oesper (67), however, concluded from equilibrium studies that

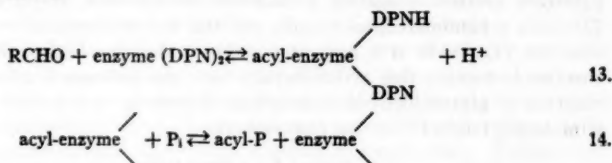
no reaction of phosphate and substrate occurs in the absence of the dehydrogenase. From a somewhat different viewpoint the question of phosphate participation has been again investigated by Velick & Hayes (68). Although phosphate is extensively bound by the enzyme and markedly alters some of its physical properties, no complex with the enzyme is essential to the enzymatic reaction, for this binding of phosphate can be completely suppressed by other anions without influencing the activity of the enzyme.

In fact, the enzyme will oxidize the substrate in the complete absence of phosphate, albeit slowly, and this has led two laboratories (65, 68) to conduct experiments providing direct evidence of an acyl enzyme intermediate. Using an excess of substrate and DPN and substrate levels of enzyme, it was observed that, in the absence of phosphate, reduction of DPN occurred in a rapid initial burst, followed by an abrupt break and slow continued reaction. The break in the curve corresponded to the reduction of two moles of DPN per mole of enzyme.

Addition of small amounts of phosphate initially or after the rapid phase of DPN reduction had occurred led to the same final equilibrium level suggesting that, in the absence of phosphate, an intermediate accumulated in amounts dependent on the concentration of the enzyme and that this could subsequently react with phosphate, releasing the participating group of the enzyme and enabling further reduction of DPN. From their results Velick & Hayes (68) have calculated the equilibrium constant for the formation of the intermediate and thus have estimated the constant for the phosphorolysis reaction. The latter is of the same order of magnitude as that for the acetylphosphate-acetyl coenzyme A (transacetylase) reaction (69) and is thus in accord with the principle of a thiolester intermediate.

Harting & Chance (70) have determined the absorption spectrum of the enzyme after reaction with acetylphosphate. The maximum in the difference spectrum is suggestive of a thiolester though somewhat displaced when compared with acetyl glutathione (238 $m\mu$ for the former and 232 $m\mu$ for the latter).

From the accumulated evidence on direct observation of an acyl enzyme intermediate and the arsenolysis reaction, it seems reasonable to conclude that the reactions



are the simplest representation of the process; more complex reaction sequences have been presented by Racker *et al.* (62, 71) and Segal & Boyer (65, 72). The former proposes the mechanism as illustrated in Figure 3 to account for the involvement of $-\text{SH}$ in the binding of DPN and the

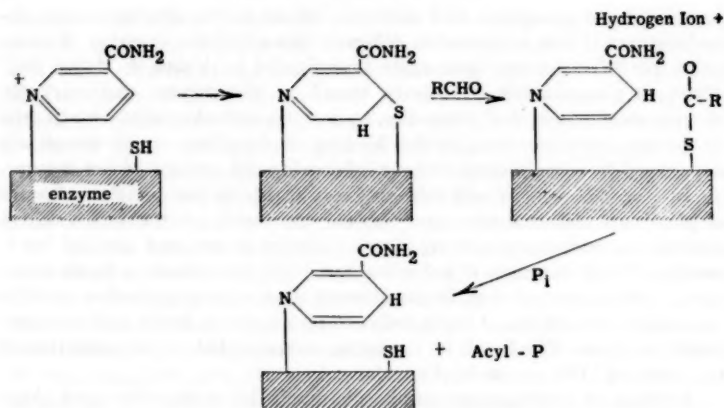


FIG. 3. A postulated mechanism for the glyceraldehyde phosphate dehydrogenase reaction.

spectral change associated with the binding. "Aldehydeolysis" of the DPN—S-enzyme link leads directly to reduction of DPN and formation of a thiol-ester intermediate. To account for all the phenomena he has proposed a secondary transfer of the acyl group to another site on the enzyme prior to phosphorolysis.

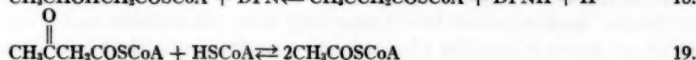
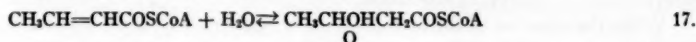
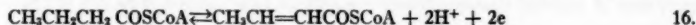
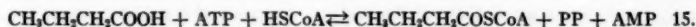
The proposed mechanism is a stimulating one in its representation of the actual oxidation-reduction process. Among the objections raised against this scheme are those of Segal & Boyer (65) who cite the independent binding of substrate associated with —SH groups and the fact that when no DPN, other than that associated with the enzyme, is present, only one-half the DPN is reduced whereas aldehydeolysis as a mechanism of substrate binding would require an equivalent of DPNH for every substrate molecule bound. The significance of the objection raised by Segal & Boyer is somewhat reduced, however, by Racker's observation on the lack of specificity of the substrate protection against *p*-chloromercuribenzoate. Boyer & Segal (65, 72) favor a hemimercaptal theory, and this has been considered by Racker also (62, 71). While it is not yet possible to decide whether either of these theories is correct, this active inquiry into the intimate mechanism of the reaction of glyceraldehyde-3-phosphate dehydrogenase is one of the most stimulating facets of current enzymology.

FATTY ACID OXIDATION

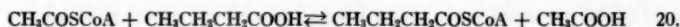
Developments in this field have been extremely rapid within the past year. The scheme of fatty acid oxidation proposed by Barker (73) on the basis of the extensive experiences of his laboratory with soluble extracts of *C. kluyveri* has now been verified in most details. However, the major de-

velopments have occurred in the identification of the various enzymatic steps in animal tissues. Keyed by the identification of "active acetate"—acetyl coenzyme A—as a thiolester of the coenzyme (74) and the divorce-ment of fatty acid oxidation from the mitochondrial level in animal tissues, [the approach to soluble enzyme systems (75 to 78)] the identification of individual enzyme steps has been accomplished in a number of laboratories.

With few reservations fatty acid oxidation may now be recognized by the following sequence of reactions:



The initiation of butyrate oxidation by formation of the HSCoA derivative suggested by Barker (73) was supported by the initial observations of Drysdale & Lardy (76) and Mahler *et al.* (78) and subsequently was demonstrated directly. Whereas in the *C. kluyveri* system this initiation is via the CoA phosphorase reaction (79, 80),



activation in the animal system appears to be so far limited to the ATP reaction (reaction 15). Isolation and characterization of the fatty acid activating enzyme catalyzing reaction 15 has been reported by Mahler *et al.* (81, 82). The enzyme will function with a variety of saturated, unsaturated, and hydroxy acids from C_4 to C_{12} . The formation of longer chain (up to C_{30}) acyl SCoA compounds by a process typified by reaction 15 has been reported by Kornberg & Pricer (83).

The enzyme responsible for reaction 16 was simultaneously identified by Mahler (81, 84) and by Lynen & Seubert (85, 86). Seubert & Lynen (86) observed that in some steps (reaction 16 and 18) the thiolesters of N-acetylthioethanolamine could substitute for the HSCoA derivatives. Using S-crotonyl-N-acetylthioethanolamine as substrate and coupling the dehydrogenase with leuco safranin or other suitable leuco dye as hydrogen donor, they were able to observe the reduction of the crotonyl compound to the butyryl derivative. Using this as an assay procedure they were able to purify the enzyme from sheep liver extracts about 80-fold. Mahler (84) obtained the enzyme of reaction 16 from beef liver in a form homogeneous in the ultracentrifuge and Tiselius apparatus. Enzyme activity in this laboratory was usually measured by reduction of a suitable redox dye such as 2,6-dichlorophenol indophenol. E_0' for the system butyryl SCoA \rightleftharpoons butenoyl SCoA is stated to be in the range of indophenol, near +0.2 v. at pH 7.0

(87). In both cases a flavoprotein was indicated. In addition Mahler observed that his preparations contained copper and that at different levels of purity of the enzyme the Cu/flavin ratio was essentially 2. Addition of butyryl SCoA caused reduction of the flavin bands as well as the band attributed to Cu. A more extensive discussion of this enzyme is presented in a later section. The product was identified as butenoyl SCoA but not specifically as crotonyl SCoA.

It will be recalled that the work of Stadtman & Barker (88) implicated vinyl acetate or an activated derivative, whereas the present work on the enzymes of animal tissues strongly suggests that crotonyl SCoA is the first intermediate in butyrate oxidation.

While the reaction of the flavoproteins of Seubert & Lynen (86) and Mahler (84) are reversible with crotonyl SCoA, it is not known whether they are inactive relative to vinyl acetyl SCoA.

In the work reported by Mahler (81) both vinyl-acetyl and crotonyl SCoA are active in reaction 17, and it was postulated that the two tautomers equilibrated through β -hydroxy butyryl SCoA. Such a mechanism requires that the enzyme be capable of adding or removing water from either of two positions. It should be observed that the shift in the double bond in the crotonic-vinyl-acetic acid system may be catalyzed by alkali (89), and this obviously does not require a hydration-dehydration mechanism. Furthermore, the equilibrium for the acids or esters is far on the side of crotonic acid (>90 per cent), so it would not be surprising if crotonyl SCoA were the only detectable form in the biological system. In the absence of more definite information excluding it, the possibility of vinylacetyl SCoA functioning as a normal intermediate in butyrate oxidation is still unsettled.

Butyryl SCoA dehydrogenase is specific for acyl SCoA from C_3 to C_8 and while similar in function to fumaric hydrogenase, also a flavoprotein (90), the two enzymes are not identical (86).

Another flavoprotein separable from the one above (87) has also been obtained from beef liver and is active only on an acyl-SCoA of chain length greater than C_6 .

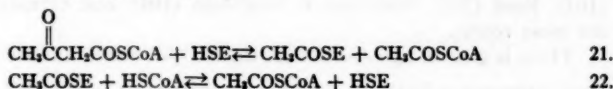
Enzyme preparations catalyzing reaction 17 have been obtained by Lynen (85), Stern & del Campillo (91) and Mahler *et al.* (81, 87). It has been referred to as crotonase, and the purest preparations of Mahler *et al.* act upon all unsaturated acyl SCoA tested from C_4 to C_{12} . As mentioned, vinylacetyl SCoA will react as well as crotonyl SCoA; however, the enzyme is not active on the *cis*-form of the latter (87).

The β -hydroxyacyl SCoA dehydrogenase of reaction 18 has been identified and purified by Lynen *et al.* (85, 92) and by Mahler *et al.* (81, 87). It reacts with hydroxyacyl SCoA from C_4 to C_{12} . Lehninger & Greville observed (93) that of the two optical forms of β -hydroxybutyrate, it is the *d* form that is involved in reaction 18. Both optical forms are oxidized by tissue extracts, of course, and both isomers will form HSCoA derivatives (87, 93); however, the *L*-isomer, sometimes regarded as the naturally occurring form,

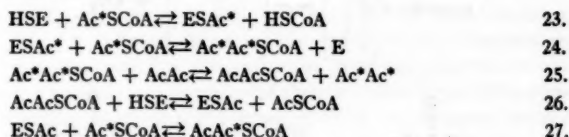
requires only DPN for its oxidation by dialyzed extracts of liver acetone powder (93).

The formation of acetoacetate from two molecules of acetyl SCoA was established by Stadtman, Doudoroff & Lipmann (94). However, the elusive acetoacetyl CoA of reaction 19 and 18 was first demonstrated by Lynen *et al.* (92) by its absorption band at 303 μ , and it was subsequently isolated by Stern *et al.* (95) and by Beinert *et al.* (87) who have also isolated and identified the β -keto acyl SCoA arising from the C_6 and C_8 hydroxy acids of reaction 18. In addition, acetoacetyl SCoA may be prepared chemically (96). The enzyme catalyzing reaction 19 has been termed β -keto thiolase (92) and is active on all β -keto acyl SCoA derivatives from C_4 to C_{12} (87).

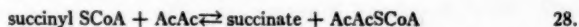
It has been suggested (81, 95, 97) that the thiolytic cleavage of acetoacetyl SCoA may be represented by



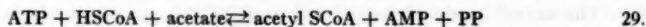
Thus the terminal two-carbon fragment in oxidation of any fatty acid would be unique in its temporary association with the enzyme. If reaction 22 occurred, it would of course be mixed with the acetyl SCoA pool but while still associated with the enzyme its participation in the reverse of reaction 21 would enjoy a greater probability and it would always contribute carbons 3 and 4 to acetoacetate. This circumstance would provide an explanation for the asymmetric labeling of acetoacetate observed by Crandall *et al.* (98) in fatty acid oxidation. Beinert & Stansly (97), employing enzyme preparations with ketothiolase activity, $\text{CH}_3\text{C}^{14}\text{OSC}^+\text{CoA}$, and unlabeled acetoacetate have reproduced these asymmetric labeling effects, suggesting in this case the following mechanism



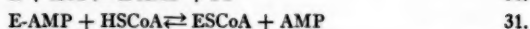
with the product of reaction 27 engaging again in reaction 25 etc., where reaction 25 is a postulated reaction analogous to reaction 28 demonstrated by Stern *et al.* (95) and Green *et al.* (99)



Mention should be made here of one further process, the "ATP-acetate reaction." This was originally represented by Lipmann *et al.* (100) as:



Recent observations (101) have indicated that the detailed mechanism may be formulated as:

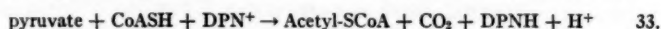


Reaction 30 was indicated by the rapid exchange of labeled pyrophosphate and ATP in the presence of the enzyme but in the absence of the other components. The inhibition of this process by HSCoA suggested reaction 31, while reaction 32 was supported by the exchange of labeled acetate with acetyl SCoA in the absence of all components other than the enzyme.

PYRUVIC AND α -KETOGlutARIC OXIDASES

The rapid development of our understanding of α -keto acid oxidation has resulted in a large number of excellent reviews of which those of Slater (102), Reed (103), Stadtman & Stadtman (104), and Gunsalus (105) are the most recent.

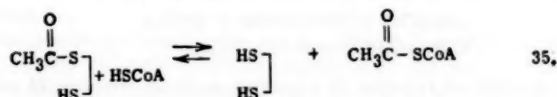
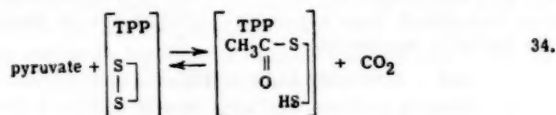
There is general agreement that the over-all reaction is identified as



for pyruvate (106, 107) and a similar sequence for α -ketoglutarate (108, 109).

In the case of pyruvate, the participation of TPP² (106, 107, 110, 111)

and lipoic acid (112 to 115) (represented as $\begin{array}{c} \text{S} \\ | \\ \text{---} \end{array}$) in the oxidation have led to postulation of the following mechanism (103, 105, 116) as seen in reactions 34, 35 and 36.



The actual mode of participation of TPP is uncertain. It is proposed (103, 105) that a two-carbon fragment at the oxidation level of acetaldehyde (carbanion) is first associated with TPP and subsequently transferred to lipoic acid forming the thiolester and a free SH. This proposal is in keeping

with the broader role of TPP arising from observations on the requirement for TPP in ketol transformations of the sugars (117, 118) and the acetoindiacetyl reactions to be mentioned later. It is further proposed (119, 120) that the coenzyme form of TPP and lipoic acid is LTPP.² (See Fig. 4.)

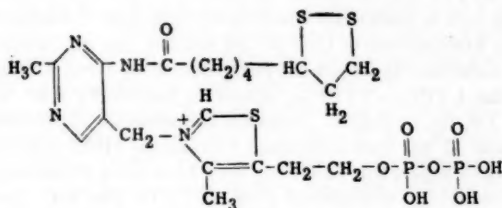


FIG. 4. Postulated structure of lipothiamide pyrophosphate.

Reed & DeBusk have presented extensive evidence in support of the participation of LTPP as the functional form of lipoic acid. Observing that yeast extract contained a number of conjugated forms of lipoic acid, they obtained by irradiation a mutant of *E. coli* that was unable to employ a mixture of TPP and lipoic acid but was able to utilize one of the conjugated forms which was subsequently synthesized from lipoyl chloride and thiamine (or thiamine pyrophosphate) and whose properties were in agreement with the above formula. Incubation of washed cells of *S. lactis*, *E. coli*, or *S. faecalis* with TPP and lipoic acid would produce the necessary activator.

Korkes *et al.* (106) observed that extracts of *E. coli* possessing pyruvic oxidase activity could be resolved into two protein fractions by ammonium sulfate fractionation, both parts being necessary for the oxidation of pyruvate. Employing the fractionation procedure of Korkes *et al.* [and Gunsalus *et al.* (111, 113)], Reed & DeBusk observed that the B fraction obtained in this way, when incubated with TPP and then heat inactivated, would supply the conjugate necessary for activity of their mutant. It was thus concluded that the function of the B fraction was that of a conjugase forming LTPP from TPP and lipoic acid bound in the B fraction.

However, there may be some uncertainty regarding the application of the procedure of Korkes *et al.* to different materials; thus, when applied to heart muscle (121) the method was, by and large, unsuccessful although a fraction of heart muscle could substitute for the B fraction of *E. coli*. This reservation concerning the fractionation procedure may be of particular significance in determining the function of fraction B, for contrary to the conclusion of Reed & DeBusk (119, 120), Gunsalus (105) maintains that nearly all of the lipoic acid is in fraction A. Furthermore, although the total contribution of the crude *C. kluyveri* extract employed as transacetylase by Reed & DeBusk is uncertain, their fraction A when supplied with LTPP carries out the complete pyruvic oxidation whereas Gunsalus (105) has ob-

served that fraction B contains the enzyme responsible for coupling reduced lipoic with DPN (reaction 36). Furthermore, he has been unable to repeat the experiments of Reed & DeBusk with the B fraction and TPP.

Reed has also observed reaction 36 in an unfractionated system employing reduced LTPP. The reduction of DPN^+ by either reduced LTPP or reduced lipoic acid is essentially irreversible (103, 105). Coupling the LTPP system to the hydrogenase of their *E. coli* mutant, the enzymatic reduction of LTPP by molecular hydrogen has been effected by Reed & DeBusk (103). The E_0' of the $\text{LTPP}_{\text{ox.}}/\text{LTPP}_{\text{red.}}$ system is essentially that of $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}$, at pH 7.0, i.e., -0.42 v. Although the isolation of reaction 36 from reaction 34 and 35 has been obtained by Gunsalus (105), reactions 34 and 35 have not been separated. However, they have been separately identified and their reversibility determined (103, 105). In this way starting with pyruvate and substrate amounts of LTPP and in the absence of DPN and CoA, Reed (103) has prepared and isolated acetyl LTPP. Reactions 35 and 36 have been observed with both lipoic acid and LTPP. Since lipoic acid satisfies the specificity of the enzymes involved equations 35 and 36 are written with it as the reactant although LTPP may be the natural functional form.

An essentially similar scheme for α -ketoglutarate is supported by observations of Hager *et al.* (111) on the fractionation of the α -ketoglutaric dehydrogenase of *E. coli* into two fractions A¹ and B, where B is the same as the B fraction of pyruvic oxidase.

To explain the behavior of the α -keto acid oxidases with artificial electron acceptors and in acyloin synthesis, Reed has proposed that the active site (for reaction 34) may be occupied by either LTPP or TPP, and that the carbanion, $(\text{CH}_2\text{C})^-$, may be generated in the presence of either LTPP



or TPP. In the case of LTPP the carbanion is transferred to the lipoic acid moiety forming acetyl lipoic, and the sequence through DPN^+ becomes obligate. If TPP occupies the site, the later reaction is not possible, but ferricyanide or other artificial electron acceptors can accept electrons from the



carbanion giving a carbonium ion, $(\text{CH}_2\text{C})^+$, which is repelled by the quaternary nitrogen of the thiazole, reacting with water to form acetic acid. The concept of "internal" acceptor (lipoic acid) in the case of LTPP and "external" acceptors in the case of TPP is extended further to cover acyloin synthesis from the carbanion and added aldehydes or the dismutation and oxidation of diacetyl as observed with purified pyruvic oxidase of heart muscle and discussed by Schweet *et al.* (122).

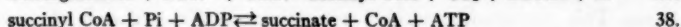
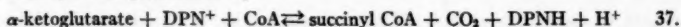
In view of the reports (111, 113) that lipoic acid is essential for the ferricyanide reaction with *S. faecalis*, Reed's generalized mechanism may require some modification.

Reed suggests that his proposed mechanism would explain the observation of Moyed & O'Kane (123) on pyruvic oxidase in *Proteus vulgaris*. Here the system was fractionated into two parts and partially purified. No CoA or lipoic acid is required. Fraction *a* oxidizes pyruvate in the presence of dyes or fraction *b*, a particulate fraction containing components of the cytochrome system. In Reed's view, fraction *a* would function as a system of the external acceptor type. For the participation of the terminal oxidase system, fraction *b*, Slater (102) has suggested that direct coupling (without DPN) occurs in the same manner as with ferricyanide.

In relation to the work of Moyed & O'Kane, Gunsalus (105) has recently applied the fractionation procedure of Korkes *et al.* (106) and Hager *et al.* (111) to *P. vulgaris*. Obtaining A and B fractions comparable with the *E. coli* system, he is led to suggest that more than one pathway for α -keto acid oxidation exists in the former organism.

Neither the pyruvic (107, 110, 121, 122) nor α -ketoglutaric (108, 109, 124, 125) oxidases of pigeon breast muscle or heart muscle have been satisfactorily fractionated in the fashion of the bacterial systems. However, the requirements for DPN and CoA have been observed (107, 110, 121, 122, 124, 125). Lipoic acid has been observed to parallel the purification of the pigeon breast muscle pyruvic oxidase, and TPP is required for this enzyme, but, no TPP requirement for the animal α -ketoglutaric oxidase has been recorded.

The enzyme (or enzymes) involved in the association of phosphorylation with the primary dehydrogenation step in α -ketoglutarate oxidation have been isolated and characterized in two laboratories (124, 125). The complete process may be represented by



Enzyme preparations catalyzing only reaction 37 have been obtained from heart muscle (124, 125) and the reversibility identified by incorporation of C^{14}O_2 into α -ketoglutarate (126).

Reaction 38 is readily reversible, and the enzyme (or enzymes) requires Mg for its activity (124, 125). In the forward direction the reaction has been measured by formation of SH, disappearance of an equivalent of Pi, and formation of glucose-6-PO₄ (with hexokinase). In the reverse direction the reaction can be observed through formation of hydroxamic acid from succinate, ATP, and catalytic amounts of CoA. In addition, an enzyme causing the hydrolysis of succinyl CoA, succinyl CoA deacylase, has been separated from the α -ketoglutaric dehydrogenase and the phosphorylation enzyme (124, 125, 127). No intermediate stages in reaction 38 have so far been identified.

ELECTRON TRANSPORT THROUGH FLAVIN ENZYMES

As considered subsequently for the case of iron porphyrin enzyme catalyzed reactions, cellular electron transport through the flavoprotein enzyme

finding does not rule out metal ion involvement since this inhibitor may not decrease significantly the two electron transfer between flavoprotein and dye acceptor systems [in this case, neotetrazolium (133)].

The nature of the "liver residue" factor of Westerfeld & Richert (134) which increases the level of liver and intestinal xanthine oxidase when fed to weanling rats, has been a subject of active investigation during the past year. De Renzo *et al.* (135) and Richert & Westerfeld (136) have demonstrated that the nutritional need for this factor can be completely replaced by the addition of molybdate (1 mg. to 1 kg.) to diets. Similar results were reported by Westerfeld & Richert (137). Totter *et al.* have shown, using radioactive Mo⁹⁹, that molybdenum is an integral, nondialyzable part of the xanthine oxidase (138). Their data suggest a molar ratio of flavin to molybdenum of 2:1. Green & Beinert (139) have arrived at the same ratio by analytical studies on milk xanthine oxidase. Xanthine oxidase was shown a number of years ago to be intensely inhibited by cyanide when O₂ was the electron acceptor but not when coupled to methylene blue (140). The presence of a metal component in xanthine oxidase probably accounts for the cyanide sensitivity of this enzyme. The enzyme also appears to be dependent on sulfhydryl groups, in view of the findings of Harris & Hellerman (141) on PCMB inhibition with cysteine reversal.

Nitrate reductase has also been shown to involve a metallic component, molybdate (142). The reduction of nitrate is of general importance to some species of bacteria (143), molds (144), and higher plants (145). For the most part, studies on this process are limited to the first step, nitrate to nitrite. Previous studies with *E. coli* (146) and plants (145) have not suggested the character of the enzymatic process, but recently Evans & Nason have isolated nitrate reductase from *Neurospora* (144, 147) and from soy bean leaves (145). They have observed that the enzyme is a flavoprotein reacting specifically with TPNH in the former case and with either TPNH or DPNH in the latter. They have also observed in preliminary experiments with preparations from soy bean leaves that TPNH is required for nitrite disappearance.

Harrison has recently described the activation of fumaric hydrogenase by ferrous ions (148). This metal ion is required in addition to FAD to restore the activity of dialyzed and alumina C γ -treated enzyme. Other metals such as Mn⁺⁺ were ineffective. Although its chemical nature is not established, succinic dehydrogenase, a nonheme enzyme, is cyanide sensitive in the one electron transfer it catalyzes between its substrate and the iron-porphyrin enzyme complex (149). Examination of the literature reveals that no all-inclusive statement involving the participation of metals in oxidations catalyzed by flavin enzymes can be made (e.g., L-amino acid oxidase is cyanide sensitive, notatin and D-amino acid oxidase are not, etc.), although such involvement may only occur in those systems which carry out one-electron transfers.

The growing evidence favoring a common link in the oxidation of succinate, DPNH, butyryl-CoA, and other substrates, when considered in con-

junction with the findings on metallo-flavoproteins described above, suggests the generalized scheme of "integrated" oxidation seen in Figure 5. In this scheme, which is in fact a combination and reiteration of similar earlier suggestions, cyanide sensitive steps involving one-electron transfers are pictured as occurring mainly in the integrated oxidation systems leading to energetic coupling with phosphokinase mechanisms, although, as indicated, such electron transfers can be observed in solubilized flavoprotein systems. The integrated and solubilized mechanisms are, however, distinguishable on the basis of their sensitivity to the usual agents which uncouple oxidative phos-

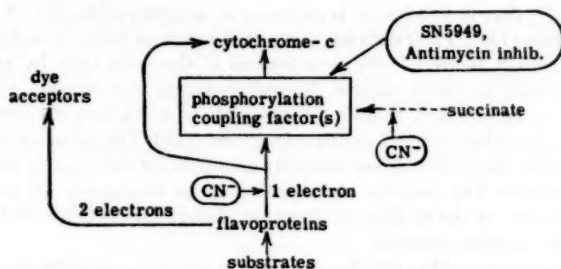


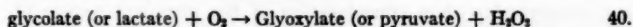
FIG. 5. Generalized scheme of "integrated oxidation" indicating the possible involvement of one-electron transfers in coupled phosphorylations.

phorylation. The scheme suggests the possibility that certain flavin-linked oxidations, hitherto considered to be independent of structural factors in cells, may, under proper conditions of study, be shown to serve as sources of biosynthetic energy through a universal coupling mechanism.

Future research in the flavoprotein field should be greatly facilitated by certain preparative procedures recently described. Christie, Kenner & Todd (150) have reported preliminary details of their total synthesis of FAD. Their final products exhibited full activity in the reactivation of D-amino acid oxidase apo-enzyme. FAD has also been isolated in almost pure (90 per cent) form by Whitby (151) who employed a chromatographic procedure on powdered cellulose. His product showed a diminution in light absorption at pH 7.0 relative to that predicted from the combined flavin mononucleotide and adenylic acid spectra. He suggests that this may be attributable to the formation of an internal complex at this pH. Dimant *et al.* (152) have also described procedures for flavin nucleotide isolation using adsorption chromatography on Florisil and partition chromatography on Celite.

Baker (153) has described an enzyme preparation from hog kidney cortex, containing at least two enzymes, distinct from lactic dehydrogenase, which catalyzes the oxidation of L- and D-hydroxy acids. The oxidations appear to be flavin-linked since the addition of riboflavin phosphate introduced a marked stimulation in O₂ uptake when added to dialyzed preparation.

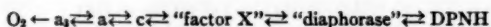
Riboflavin phosphate was also shown to be the prosthetic group of glycolic acid oxidase, partially purified (500-fold) from spinach leaves by Zelitch & Ochoa (154). Physical examination indicated at least 75 per cent purity. The isolated enzyme carries out the reaction:



The hydrogen peroxide produced reacts nonenzymatically with glyoxylic (or pyruvic) acid to form formic (or acetic acid), CO_2 , and water. The relatively low dissociation constants ($3.8 \times 10^{-4} M$ and $2.0 \times 10^{-3} M$) of the enzyme leads the authors to suggest its usefulness for the analytical determination of glycolate and L-lactate. Glycolic acid oxidase was also studied by Kenten & Mann (155) who obtained results identical with those of Zelitch & Ochoa.

CATALASE AND PEROXIDASE CATALYZED OXIDATION-REDUCTIONS⁴

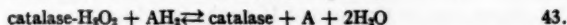
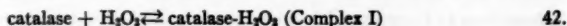
Although not yet established as a general mechanism, the oxidation of ferrocytochrome-*c* by peroxidase appears to account for a considerable share of electron transport in intact yeast cells under certain conditions. The studies of Chance (156) show that in the presence of glucose the quantity of peroxidase-Complex II formed with CH_3OOH in aerated, starved yeast cells is considerably less than without added substrate. This observation is interpreted as an indication of augmented reduction of cytochrome-*c* by electrons derived from the oxidation of glucose and its products through the electron transport system.



Support for this conclusion was obtained upon the addition of antimycin A, which presumably blocks electron transfer through "factor X" to cytochrome-*c*. In this case, the addition of glucose no longer caused an appreciable increase in peroxidase turnover.

It was also found that the addition of CH_3OOH to anaerobic yeast cells resulted in the nearly complete oxidation of DPNH and reduced flavo-proteins as well as ferrocytochrome-*c*.

Chance has continued his *in vivo* studies on the catalase-catalyzed reactions of *Micrococcus lysodeikticus* (156). In accordance with the reactions:

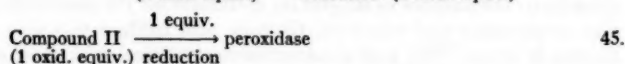
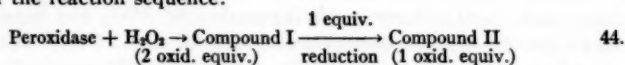


the disappearance of catalase-Complex I is observed as respiring cells deplete their surroundings of O_2 , presumably as a result of the progressive de-

⁴ Since the exact chemical nature of the peroxide-peroxidase intermediate is not yet established, the terms "complex" and "compound" are used as they appear in the articles quoted.

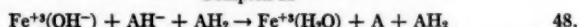
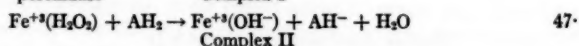
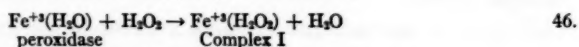
crease in metabolically produced peroxide. Addition of 2 mM formate (as AH_2) reduces the steady-state Complex I concentration to about 50 per cent through reaction 43 above.

During the past year a large number of papers have been published which are concerned with a consideration of the chemical nature of peroxidase intermediate compounds. Careful titration studies (157, 158) appear to have established the reaction sequence:



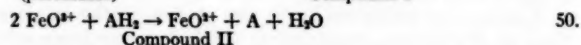
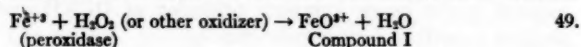
However, the valence state of the iron atom in the peroxidase molecule appears still to be the basis for some disagreement between workers in this field. George (159) has presented evidence for the production of Compound II and mixtures of Compounds I and II by the action of a number of oxidizing agents (e.g., HClO , NaClO_2 , KIO_4) on peroxidase. Chance (160) has confirmed and extended these findings by showing that the reaction kinetics of Compound I formed in such a manner are similar to those of Compound I formed by H_2O_2 .

Both George and Chance have suggested general equations to account for the single-electron reduction reactions relating peroxidase Compound I and Compound II. Chance's reactions (156) are as follows:



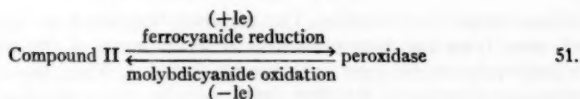
They avoid the postulation of unknown higher valence states of iron, but do not, however, account for the observation that Complexes I and II can be formed by oxidizing agents other than H_2O_2 .

George (157) suggests that the peroxidatic reactions take place as follows:



where iron in Compound I exists either as Fe^5 or in some equivalent structure as shown, and in Compound II as the ferryl ion.

He has suggested (159) that the description of peroxidase-catalyzed reactions in terms of Michaelis-Menten "enzyme-substrate complexes" is inappropriate in view of the apparent nonessentiality of H_2O_2 for such oxidations. The formation of Compound II from horseradish peroxidase by single-electron transferring oxidizing agents (161) (chloroiridate and molybdicyanide) adds support to his suggestion.



Further work is clearly needed to establish definitely the chemical nature of the intermediary molecules formed during peroxidatic reactions.

Polis & Shmukler (162) have isolated crystalline lactoperoxidase employing an elegant displacement-chromatographic method during the purification procedure. A highly purified yeast catalase, prepared by Brown (163), showed the same instability observed earlier by Deutsch (164) in the case of erythrocyte catalase.

ELECTRON TRANSPORT AT THE CYTOCHROME LEVEL

The almost complete inhibition of cellular respiration by agents which interfere with the normal functioning of cytochrome oxidase (165) or of other cytochrome components of the respiratory chain (166, 167, 168) has long indicated the obligatory role of these enzymes in the production of biosynthetically useful energy. During the past year, however, some successful beginnings have been made in a number of laboratories to separate and recombine the components of the terminal oxidase complex and its closely related tributary, the flavoprotein-containing DPNH oxidase system.

Stotz and his colleagues Clark, Widmer and Wenfeld have continued the excellent studies from the Rochester laboratory on solubilized succinoxidase components (personal communication). The "SC factor" described contains succinic dehydrogenase but is free from cytochromes-*c*, *a*, and *a*₃. Upon ultracentrifugation, SC factor activity was found to sediment with the red, spectroscopically characterized cytochrome-*b*, and with a second heme-protein, termed cytochrome-553. The yellow supernatant material, which separated into two ultracentrifugal peaks, showed a succinic dehydrogenase: SC factor ratio many-fold greater than the uncentrifuged SC factor preparation, indicating the nonidentity of succinic dehydrogenase and cytochrome-*b*. These direct results confirm and establish this nonidentity, previously indicated by the cyanide inhibition studies of Tsou (149), the steady state analyses of heart muscle preparations by Chance (169), and the lack of correlation between succinic dehydrogenase activity and cytochrome-*b* content observed in studies on the "poky" strain of *Neurospora* (170).

Together with their earlier cytochrome oxidase preparation and added cytochrome-*c*, the new fractions of Stotz *et al.*, have permitted the "reconstruction" of a succinic oxidase system with a turnover number (measured for cytochrome-*b*) of 1070 gm. atom of cytochrome-*b* iron/min.

Although not implicitly stated, the term "SC factor" as used in the Rochester studies appears to be a broad one, including the entire sequence of components that are necessary to link succinate oxidation to cytochrome-*c*. Indeed, the experiments indicate rather clearly that "SC" activity occurs in direct proportion to the concentration of cytochrome-*b* during chemical

or centrifugal fractionation. The material designated as "cytochrome-553" appears, from the data presented, to fulfill some of the requirements for a linking-factor between cytochromes-*b* and *c*. Thus the addition of the alkyl naphthoquinone SN5949 (166) permits the reduction of "553" and cytochrome-*b* by succinate, but prevents reoxidation of these components by cytochrome-*c* in reconstituted system. The approximate potential of "553," somewhere at or above 0.21 v. (as indicated by dye reduction studies) is in accord with its suggested position in the cytochrome sequence.

By combining this reconstructed system with the nonpyridine nucleotide linked chicken liver "xanthine dehydrogenase" (171) or α -glycero-phosphate oxidase preparations, respiring systems were obtained. The latter reconstructed preparation, in the presence of cyanide, reduced cytochrome-*c* but not *b*. Antimycin A and SN5949 prevented this reduction indicating the possible presence of a similar cytochrome-*c* linking factor in both the "SC factor" complex and the α -glycero-phosphate oxidase.

Further studies on the effect of lecithinase A on the terminal oxidase system have been reported by Nygaard (172). His findings indicate that such treatment destroys a factor or factors involved in electron transport between succinic dehydrogenase or DPNH and cytochrome-*c*. Of especial interest was his observation that, whereas in liver particulates lecithinase inactivates "succinate-cytochrome-*c* reductase" but not DPNH cytochrome-*c* reductase, whereas in heart preparations the reverse was true. Soluble DPNH-cytochrome-*c* reductase, diaphorase, and succinic dehydrogenase were unaffected or only slightly so.

The findings of Nygaard and of Stotz *et al.* are of particular interest when considered together with the studies on solubilized cytochrome-*c* reductases described in a previous section. The involvement of obligatory, antimycin A- and SN5949-inhibited components, associated in some manner with lecithinase-sensitive materials [see also Ball & Cooper (173)] in the particulate systems is in strong contrast to the direct mechanism of electron transfer from DPNH to cytochrome-*c* in the solubilized systems (e.g., 128). Thus thermodynamically feasible electron transfers which may occur readily in solubilized preparations through free collision appear to be subject to rigid sequential electron transfers as a result of geometric restriction in the integrated oxidation "unit process." The philosophically minded worker in this field should perhaps mount on his laboratory wall Pallades' splendid electron-microscope pictures (174) of mitochondria with their provocative indication of protein monolayers.

Characterization of succinic oxidase components.—Using novel spectrophotometric techniques, Chance has determined the molecular extinction coefficient for the bands of the carbon monoxide compounds of cytochrome-*a₃* in muscle and yeast and of cytochrome-*a*, in *Acetobacter pasteurianum* (11–12 $\text{cm}^{-1} \times \text{mM}^{-1}$ at 589 $\text{m}\mu$) (175). These studies, as well as those of Smith (176), suggest that the view that cytochromes-*a₃* and *a* are invariably

associated is incorrect. Smith, for example, reports that *Staphylococcus albus* shows the bands for cytochrome-*a* without any evidence for a_3 . In *B. subtilis* the ratio of *a* to a_3 was found to be considerably greater than in yeast. Wainio (177) has suggested that cytochrome- a_3 may be the only enzymatic component between cytochrome-*c* and O_2 on the basis of his studies with the "solubilized" a_3 preparation. The observed coupling may be another example of the sort of nonintegrated electron transfer which occurs with the solubilized pyridine nucleotide-cytochrome-*c* reductases, although, as he suggests, the spectrum known as cytochrome-*a* may indeed be attributable to a partially reduced form of a tetrapolymer of heme-protein in which each contributes one electron to the reduction of one mole of oxygen. Ball & Cooper (178) have presented data which suggest the reduction of oxygen through a complex of three cytochrome-*a* porphyrins and one cytochrome oxidase porphyrin.

Cytochrome-*c* reduction in a crude succinoxidase system was found by Dickman (179) to be inhibited by ferri-xanthyl cytochrome-*c*, which, however, did not inhibit ferrocytochrome-*c* oxidation and, indeed, can itself be oxidized in such preparations by O_2 . Brodie & Gots (180) described the use of a nitrofur, furacin, as an electron acceptor in the oxidation of DPNH by diaphorase, diverting electrons from the normal pathway through cytochrome-*c*. Margoliash (181) has reported on certain improvements in the chromatographic procedure of Palés & Neilands (182) for the preparation of "0.43 per cent iron" cytochrome-*c*. His method yields stable dry products in either the ferrous or ferric form.

Several studies on the inhibition of succinic dehydrogenase have appeared during the past year. Thorn (183) has analyzed the kinetics of succinate oxidation by this enzyme in the presence of malonate and concludes that the relative affinities of malonate and succinate are in the ratio of 3:1. Experimentally, the ratio K_m/K_i varied in his experiments from 4.7 to 60, depending on the nature and concentration of the electron acceptor system employed. Dietrich *et al.* (184) studied the inhibitory effect of 32 analogues of succinic acid and found in particular that acetylene dicarboxylic acid inhibited in a competitive manner.

ELECTRON TRANSPORT AND PHOSPHORYLATION

The term "oxidative phosphorylation" is universally employed to describe the generation of biosynthetically useful phosphate bonds coincident with the oxidation of a variety of metabolites. In general, two stages of the process may be recognized, one involving a derivative of the substrate (substrate level phosphorylation) and the other associated with the transfer of electrons through the flavin-iron porphyrin respiratory chain (electron transport phosphorylation).

Methods.—Commonly used methods of measuring oxidative phosphorylation involve either a determination of the decrease in inorganic phosphate

with or without a trapping agent (creatine kinase or hexokinase) or a spectrophotometric measurement of the ATP formed employing the hexokinase-"zwischenferment"-TPN system.

Slater (185) has recently introduced a procedure which may possess some advantages over those previously employed. In common with the "zwischenferment" method, this procedure measures the product formed rather than the reactant disappearing. The system involves part of the glycolytic reactions from glucose-6-phosphate through the glycerophosphate dehydrogenase reaction. According to this, two equivalents of DPNH disappear for every $\sim P$ present initially. Thus it is somewhat more sensitive than the glucose-6-phosphate dehydrogenase technique.

The use of P^{32} equilibration to deduce rates of phosphate uptake and P:O ratios has been adopted by a number of investigators (186 to 189). One criticism of this procedure has been suggested by Korkes (190). In a reversible process such as the succinyl SCoA-ATP reaction (see p. 35) it is possible that ATP and inorganic phosphate could equilibrate in the absence of oxidation although requiring, as in this case, an oxidizable component. Relative to this example, α -ketoglutarate would have to undergo some oxidation in order to initiate the process.

While the values given by Krebs *et al.* (187) for the P:O ratio of α -ketoglutarate oxidation do not seem subject to this error insofar as they are in accord with observations by other methods, the high initial values for succinate (somewhat greater than 2) might be explained on this basis.

By the same method Bartley (188) has arrived at a figure of four for the P:O ratio in the oxidation of pyruvate to acetate by particulate preparations from sheep kidney. This figure is considered to be in agreement with that obtained with the analogous oxidative decarboxylation of α -ketoglutarate. However, there are some fundamental differences in the known mechanisms of substrate level phosphorylation in these two systems (see p. 33 and 35). Whereas the oxidation of α -ketoglutarate yields succinyl SCoA which reacts enzymatically with ADP and inorganic phosphate to give ATP, succinate, and HSCoA, the product of pyruvate oxidation is acetyl SCoA which would, by known pathways, react with AMP and pyrophosphate to give ATP. The only known process involving inorganic phosphate (occurring in bacteria) requires initial formation of acetyl phosphate, which appears to be unnatural in animal systems. Therefore, it is not immediately clear how inorganic phosphate finds its way into a mechanism presumed to require pyrophosphate and leading to such high efficiencies as those reported by Bartley.

Oxidative phosphorylation in "nonmitochondrial" systems.—It has been a common experience that the ease, extent, and efficiency of phosphorylation associated with electron transport is a function of the structural integrity of the cellular particulates. It is therefore encouraging to view some possible weakening of this structural barrier. Green *et al.* (191) have observed phosphorylation during succinate oxidation by heart muscle particles altered

to an indeterminate degree by treatment at low pH and by freezing. Pinchot & Racker (58) reported the occurrence of oxidative phosphorylation associated with DPNH oxidation by extracts of *E. coli* prepared by sonic vibration. It was not possible to obtain phosphorylation by direct addition of DPNH, but the DPN was reduced in the incubation mixture with alcohol and alcohol dehydrogenase. As a consequence it was necessary to exclude the substrate level phosphorylation associated with acetaldehyde oxidation (see p. 24), and also to suppress glycolysis. The system was somewhat atypical since it was cyanide insensitive and exhibited no DNP² effect.

More recently Pinchot (192) has described a "soluble" enzyme system from *Alcaligenes faecalis*. The organism proved to be a fortunate choice, for anticipated sources of substrate level phosphorylation, acetaldehyde and glucose-6-phosphate, were inert in the system. The extracts prepared by sonic vibration were fractionated to give three components: two enzyme fractions and a heat-stable, nondialyzable component. The oxidase fraction appears to have been an initially highly dispersed particulate material which also possessed some associated phosphorylation. The addition of a "soluble" enzyme fraction markedly stimulated oxygen uptake without altering the phosphate uptake. The addition of the heat-stable fraction alone also stimulated oxygen uptake. Addition of both of the latter fractions to the oxidase led to stimulation of both oxygen uptake and phosphorylation. However, the P:O ratios observed for the oxidase alone and for the "complete" system were the same. Phosphate uptake was observed when DPNH was added directly to the medium as well as with the alcohol-alcohol dehydrogenase system. Like the *E. coli* system, the *A. faecalis* preparations were insensitive to DNP, and on the basis of present information it is not possible to conclude to what degree the systems observed in these bacterial extracts are equivalent to those obtained in animal particulate preparations.

Thyroxin and triiodothyronine.—Although some objections have been raised (186, 193) against the conclusions of Martius & Hess (194) relative to *in vitro* uncoupling of phosphorylation by thyroxin, experimental observations leading to similar conclusions have now been presented by others (193, 195). Hock & Lipmann (195) demonstrated a thyroxin effect on P:O ratios by brief preliminary incubation of liver mitochondria of normal Syrian hamsters with thyroxin. The effective levels were similar to those at which DNP exerts its effects, i.e., 1×10^{-5} to 1×10^{-4} molar, considerably higher than those required for *in vivo* effects of thyroxin. They were not able to observe uncoupling with rat liver mitochondria. Preliminary results with triiodothyronine indicated that this substance was also effective.

Maley & Lardy (193) have observed a depression of P:O ratios in the presence of thyroxin using rat kidney mitochondria. These effects were enhanced by malonate. Curiously, however, the only oxidizable substrate for which such effects were reported was glutamate whereas no effect was obtained with α -ketoglutarate.

It was observed by Maley & Lardy that wherever depression of the P:O

ratio occurred with thyroxine, oxygen uptake was also suppressed. Triiodothyronine, on the other hand, decreased the P:O ratio at levels where no significant influence on oxygen uptake was observed.

Dinitrophenol and the mechanism of electron transport phosphorylation.—Among the chemical agents and modifying conditions interfering with the phosphorylation mechanism without significantly influencing the oxidative processes, DNP has received the greatest consideration. Recent attention has been directed at the parallel increase in adenosinetriphosphatase activity (196, 197, 198). Uncertainty still exists concerning the extent to which phosphate uptake in the presence of DNP is to be related to the increased rate of ATP breakdown. The latter in turn has raised the question of changes in the "availability" of the adenosinetriphosphatase to external ATP. A number of experimentally supported arguments suggest that the lack of permeability is not a serious problem. Thus the myokinase of the mitochondria seems fully accessible to the nucleotides (199). Although the enzymes of the particle appear to have an advantage in reaction with ATP formed in the particle in contrast to external enzyme systems (200), the activating effect of phosphate and phosphate acceptors on oxygen uptake (200, 201), the stimulating effects of DNP on respiration (200), and the high efficiencies of the oxidative phosphorylation reactions indicate that the low adenosinetriphosphatase activity of the undamaged particle is not simply attributable to permeability factors.

It would likewise seem evident that the influence of DNP on incorporation of P^{32} (202) into ATP or on the turnover of oxygen in O^{18} -labeled phosphate (203) during oxidation would exclude the possibility of attributing all the effect of DNP to increased rate of ATP destruction.

Recognizing, however, that the decrease in phosphorylation and increase in adenosinetriphosphatase activity are in some way related, the DNP-activated adenosinetriphosphatase is regarded as somehow different from that obtained by other means (196, 198). It has been suggested (204) that the inactive or "latent" adenosinetriphosphatase of mitochondria functions in the phosphorylation process and a similar proposal is the basis of the mechanism of the DNP effect suggested by Lardy & Wellman (198). In this scheme (see Fig. 6) a phosphorylated component of the system reacts with DNP and subsequently with water to provide an internal phosphate cycle. The direct reaction of $E \sim P$ with water [reaction (d)] is regarded as a result of treatment affecting the morphological integrity of the particle. With reactions (a), (b), and (c), proceeding readily in intact mitochondria both "uncoupling" and adenosinetriphosphatase would be accounted for. Such a proposal implies that the DNP effects could be reversed by removing the DNP and, indeed, both the adenosinetriphosphatase effect as well as the "uncoupling" have been more or less completely reversed under some circumstances, whereas the changes induced by other means such as aging, have not.⁵

⁵ Unpublished observations of R. Kielley and W. Kielley.

However, the proposal implies also that the ADP inhibition of the adenosinetriphosphatase prepared from disintegrated mitochondria (205) [representing reactions (a) and (d) presumably] might function through "backing up" reaction (a). Unfortunately, it has not been possible to introduce ADP³² into ATP by this process.⁴

Lee & Eiler (189) have proposed a somewhat different scheme for the mechanism of action of DNP. Its principal difference from the one given above is the involvement of a cycle of oxidation-reduction for regeneration of the component comparable to E in the mechanism of Lardy & Wellman. This would exclude an integration of the uncoupling and adenosinetriphosphatase effects of DNP.

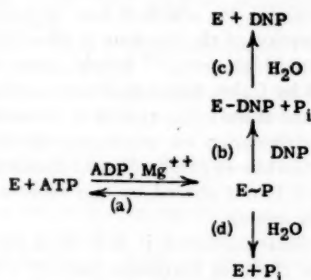


FIG. 6. A postulated reaction sequence accounting for the activation of adenosinetriphosphatase by dinitrophenol.

It would appear that both of these "short circuited" phosphate cycles are inconsistent with the results of studies on the turnover of oxygen in O¹⁸-labeled inorganic phosphate during electron transport phosphorylation and the inhibition of this turnover by DNP reported by Cohn (203). Employing this means of tracing the course of phosphate in this process, she has presented certain qualifications that must be met by any hypothesis concerned with the mechanism of electron transport phosphorylation. These studies revealed that the phosphorylation process must involve (a) a P—O bond cleavage, (b) a large number of reaction cycles per phosphate molecule, and (c) a process incorporating water in order to account for the dilutions obtained. In addition the O¹⁸ exchange required the presence of Mg and AMP, suggesting that a phosphate acceptor is required and possibly, through the Mg requirement, that a transphosphorylation process is involved directly or indirectly. The possibility exists that these components are not directly associated with the exchange reaction but with maintenance of a metabolically intact system throughout the incubation. The possibility of a direct reaction of ATP as the primary one involved in this O¹⁸ turnover was excluded, and the exchange was dependent on the oxidation of substrate by

particulate preparations exhibiting coupled phosphorylation. However, it was observed that some O^{18} was lost from the phosphate either aerobically or anaerobically with liver mitochondria in the absence of substrate.

The qualifications presented above appear to require some sort of reversible hydration reaction of an intermediate or transient state. Components of the electron transport system which, in the "resting" state, exist, almost entirely in the oxidized form whereas in the "active" system they participate as reduced forms in a reversible hydration and phosphorylation reaction might satisfy the requirements of Cohn's results. The process visualized would be somewhat analogous to the mechanism proposed by Warburg & Christian (66) for the triose phosphate dehydrogenase reaction. The analogy is perhaps a poor one since it is now regarded as a highly improbable mechanism for the situation for which it was originally proposed. On the other hand, no equilibration of the oxygens of phosphate with water in the phosphorylysis of other "high energy" bonds occurring in substrate level reactions was observed by Cohn. Some mechanism different from that operating in phosphorylations of the latter type is therefore indicated.

Although further elaboration on mechanism is plainly speculation at present, it may be worthwhile to emphasize one characteristic of the electron transport system which is not shared by the substrate level reactions on which we must lean for models.

In the case of succinate oxidation it is evident (see p. 40) that all the recognized links in the chain of reactions through which phosphorylation must arise are iron porphyrin components. Investigations (see p. 37) in connection with the metal ion components of flavoprotein enzymes suggest that stepwise, one electron oxidation-reductions may be of general importance. It is not unlikely therefore that electron transport phosphorylation in general may require the participation of coupling mechanisms geared to one electron transfer.

Available evidence does not permit us to visualize a phosphorylation reaction involving the valence change of iron in the heme groups or in a semi-quinone type of reaction, but it is these recognizable one-electron processes that distinguish the terminal oxidase systems from reactions on the substrate level and incorporate the peculiarities of phosphate turnover in electron transport.

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PROTEOLYTIC ENZYMES^{1,2}

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Some aspects of the chemistry of proteolytic enzymes and of their mode of action have been discussed in three reviews (1, 2, 3).

INHIBITION OF PANCREATIC ENDOPEPTIDASES

By organic phosphates.—Much attention is still being devoted to the inhibition of chymotrypsin and trypsin by organic phosphates, particularly diisopropylfluorophosphate (DFP).³ The inhibition is brought about by the replacement in the enzymatic proteins of a mobile hydrogen by a single alkyl-phosphoryl radical. It would be highly interesting to know the site (that is to say, the amino acid side chain) at which this replacement occurs. After digestion of radioactive diisopropylphospho- α -chymotrypsin (DP- α -chymotrypsin)³ by pepsin and trypsin and fractionation on Dowex-50 columns, only one well-defined band could be obtained. It contained orthophosphoric acid with 25 per cent of the total P³². A second radioactive band appeared (31 per cent of the total P³²), either when the digest was further hydrolyzed by 2N HCl, or when the protein was directly treated with 2N HCl. The material of this second band has been identified with O-phosphoryl serine by a number of means [Schaffer, May & Summerson (4)]. The same compound (containing 46 per cent of the total radioactive phosphorus) has also been identified in a partial hydrolysate of DFP-inhibited eel cholinesterase (5). The fact that two esterases, so different in many respects, are both inhibited by organic phosphates and that both give rise to O-phosphoryl serine on partial hydrolysis, is probably of the greatest importance. But we must refrain thus far from postulating that the inhibition is actually brought about by the specific blocking of one serine residue in the enzyme molecule. The liberation of such a large quantity of inorganic phosphate during hydrolysis is a serious warning which cannot be neglected. When the active center of an enzyme is dealt with at short range, very peculiar reactions are likely to take place. The phosphorylated radical is obviously unstable and can migrate during the breakdown of the protein. In this case the serine residue would not be the primary point of attachment of the radical, but only the comparatively stable end of an intramolecular travel. Furthermore, the whole spatial arrangement around the "active center" of the enzyme is likely to play a role during the inhibition. In this connection, investigations by Wagner-Jauregg & Hackley (6) on model substances may be of interest. Hydrolysis of DFP and of

¹ The survey of the literature pertaining to this review was completed in November 1, 1953.

² The following abbreviations have been used in this chapter: DFP for diisopropylfluorophosphate; DCIP for diisopropylchlorophosphate; DP- α -chymotrypsin for diisopropylphospho- α -chymotrypsin.

diethylfluorophosphate is accelerated by histidine and imidazole. The catalytic effect is greatly enhanced by the copper chelates of ethylenediamine-*o*-phenanthroline and α, α' -dipyridyl (7). It is tentatively explained by the formation of a quaternary ammonium compound, $\text{N}^+ - \text{PO}(\text{OR})_2$, with one of the cyclic nitrogens, in which the reactivity of the phosphoryl radical is high. In a protein possessing the right configuration, the same catalytic effect could occur, the histidine nucleus permitting the combination of the activated radical with a normally "unreactive" residue. This hypothesis is in accord with the fact that chymotrypsin, when deprived of one of its histidine residues by photooxidation, no longer reacts with DFP (8). Furthermore, a possible contribution of histidine to chymotrypsin- α active center has been sometimes evoked. On the one hand, the binding between the enzyme and its substrate may be ascribed to ionized imidazole groups [Doherty & Vaslow (9)]. On the other hand, total inactivation by photooxidation occurs when 1 mole of histidine out of 2 and 2.6 moles of tryptophan out of 6 are destroyed [Weil & Buchert (10)]. Finally, the inactivation noted some years ago by Sizer (11) and attributed to a substitution in phenol rings may as well be ascribed to a substitution in the imidazole.

Another method for studying the same problem is based on the observation that DFP is a much more potent inhibitor than its chloro analogue (DCIP).² Since inhibition occurs in slightly alkaline solution, it is to be expected that the "right" grouping will react more easily with DFP than with DCIP in the same conditions. Actually, DCIP is more reactive in nonpolar media (12). In aqueous solutions, DFP does not react readily with any of the amino acid side chains, a notable exception being the phenol group of tyrosine (13), with which it combines more rapidly than DCIP (14). It is clear, however, that this second method is not as promising as the first and that it does not take into account the possible catalytic effects mentioned above.

Cunningham & Neurath (15) have reported that the crystallization of chymotrypsin- α is inhibited by diethyl-*p*-nitrophenylphosphate; they found it to be 99.6 per cent inactive. This derivative is very probably diethylphospho- α -chymotrypsin, since the inactivation runs parallel with the liberation of one equivalent of *p*-nitrophenol [Hartley & Kilby (16)]. It can be reactivated to the maximal extent of 40 per cent (esterase activity) by 2*M* hydroxylamine at pH 6 to 8. DP- α -chymotrypsin is only 5 per cent reactivated under the same conditions. The diisopropyl derivative of cholinesterase is also more slowly reactivated than the diethyl derivative, the difference being attributed to a steric effect [Wilson (17)]. These examples of reactivation are highly interesting, since they show that the inhibited enzymes have suffered no other major changes than those brought about by the chemical reaction under investigation and since they may help towards a better understanding of the inhibition mechanism. Wilson (17) suggests that this mechanism parallels the mechanism of normal enzymatic activity. If it is true that an esterase acting on a carboxylic acid ester sets free an alcohol

and binds an acyl radical, one can postulate that water rapidly hydrolyzes the acylated protein, thus regenerating the active enzyme and giving rise to a carboxylic acid. When the same esterase is mixed with an organic phosphate, a phenol (nitrophenyl phosphate) or halogeno acid (fluorophosphate) is formed, and the protein binds an organo-phosphoryl radical. But this radical is far more stable and to be eliminated requires an acceptor more powerful than water. Thus, the carboxylic ester is an ordinary substrate and the phosphate, an inhibitor. This interesting concept is taken up again under another form by Hartley & Kilby (16) and Gorini & Felix (17a), who consider the organic phosphates as peculiar substrates on which the enzymes can act only once.

Chymotrypsin- α , inhibited by the insecticide O,S-diethyl-O-*p*-nitrophenylthiophosphate or its oxygen analogue, is not spontaneously reactivated by prolonged standing in aqueous solution. The contrary is true when cholinesterase from human red blood cells or electric eel is inhibited by the same reagent [Jandorf & Crowell (18)].

The fractionation which occurs during the crystallization of DP- α -chymotrypsin, already mentioned by Jansen *et al.* (19), has been confirmed [Desnuelle, Röver & Fabre (20, 21)] and extended to DP-trypsin [Röver, Fabre & Desnuelle (22)]. The extra N-terminal residues which are eliminated during this crystallization, probably belong to some peptides tightly bound by the active center. When the center is blocked, the peptides can be desorbed. Sorm, Keil & Rychlik (23) have also noticed that DP- α -chymotrypsin contains fewer N-terminal residues than chymotrypsin- α itself. However, they express the view that chymotrypsin- α crystals are composed of several active proteins with different N-terminal residues. One of these proteins (with the more apolar end groups) would preferentially crystallize in the presence of DFP. Whatever the truth may be, it remains that chymotrypsin- α and trypsin can no longer be considered as chemically "pure" proteins. The DP-enzymes represent, therefore, a convenient material for chemical and physico-chemical studies, since they are more stable and purer than the active enzymes.

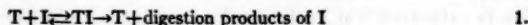
Natural trypsin inhibitors of protein nature.—Green & Work (24) have described a method for crystallizing pancreatic trypsin inhibitor from pancreas residues of the insulin industry. The main steps of the procedure include sulfuric acid extraction, precipitation in 80 per cent saturated ammonium sulfate, obtaining of a 25 per cent trichloroacetic filtrate, fractionation in saturated NaCl solution by varying the pH from 5.8 to 3.0, electrodialysis, NaCl precipitation of the catholyte at pH 3, crystallization of the trypsin-trypsin inhibitor compound in the usual way and crystallization of the inhibitor itself from saturated MgSO_4 between pH 6.5 and 3. The product is homogeneous in the solubility test, and its general composition and properties remain constant from batch to batch. But its activity towards trypsin differs rather widely, the more active pancreas giving the more active crystals. It contains all the commonly-occurring amino acids except tryptophan

and histidine and seems to have one N-terminal arginine. It crystallizes as a salt at pH 3.

The inhibition of trypsin by proteins represents an interesting biological problem. It gives also a highly specific example of protein-protein interaction at the end of which the protein substrate is not attacked and the protein enzyme is inhibited. It has been mainly investigated by determining the residual tryptic activity in trypsin-inhibitor mixtures of various composition [Green & Work (25)]. The ultracentrifuge can also be used, since the complex has its own molecular weight, but the results are somewhat complicated by boundary anomalies [Sheppard & McLaren (26)]. The most striking fact revealed by all these studies is undoubtedly the strong pH dependence of the rate and extent of the reaction between trypsin and its protein inhibitors. With the pancreatic inhibitor (25), trypsin reacts quickly between pH 7 and 10.4, but no interaction occurs at pH 3 to 4 or 10.4. The complex of trypsin with the soja inhibitor (27) is stable between pH 3.6 and 10.4, but dissociates into its components below pH 2.9. This fact brings another confirmation to Kunitz's theory, according to which the proteins are mainly linked by electrostatic bonds. In the case of the pancreatic inhibitor, whose isoelectric point is very high [higher than 10 (24)], these bonds must be strong enough to counterbalance the repulsion between two proteins carrying a net positive charge.

Green & Work (25) have calculated for the dissociation constant at pH 7 of the pancreatic inhibitor-trypsin complex a value very similar to that already found by Grob (28), $2 \times 10^{-10} M$ and $6 \times 10^{-10} M$, respectively. It is hardly necessary to recalculate this value with the new molecular weight of trypsin, since the experimental errors during the determination of the constant are probably high. Nevertheless, this constant is much lower than the Michaelis constant for trypsin with a protein substrate (about $10^{-3} M$). The competitive nature of the inhibition is therefore difficult to ascertain. However, it seems competitive with both pancreatic and soja inhibitors, when synthetic ester substrates are used (29).

According to Laskowski & Wu (29a), Kasal's inhibitor is slowly digested by trypsin. Therefore, it only provides a "temporary" inhibition. Two explanations can be given for this interesting phenomenon: either trypsin (T) is still slightly active in the complex (TI) trypsin-trypsin inhibitor (equation 1); or trypsin can act upon TI by forming a Michaelis-Menten complex TIT (equation 2).



The second explanation is favoured by the authors, since the rate of the digestion seems to be a function of both (TI) and (T). Laskowski & Wu suggest that temporary inhibition of trypsin may be a safeguard for the enzyme against self-digestion. Another inhibitor for trypsin, ovomucoid, is also hydrolyzed in the presence of calcium ions (78).

The molecular weight of the complex, trypsin-soja inhibitor, has been found to be 41,000 by ultracentrifugation (26). If one assumes that the molar proportions of the two components in the complex are 1:1, which seems to be still justified even though a new molecular weight of 21,000 to 24,000 is now attributed to trypsin (see below), the molecular weight of the inhibitor remains 17,000 to 20,000, a value in good agreement with Kunitz's previous figure of $24,000 \pm 3,000$. Under the same assumption, the molecular weight of the colostrum inhibitor (30) would be 65,000. For the pancreatic (24) and lima bean (31) inhibitors, lower values have been directly determined (about 9,000). The molecular weight of ovomucoid is 28,000.

According to Jansen, Jang & Olcott (32), DP-trypsin is still able to combine with its natural inhibitors, although the combinations are weaker than those with the active enzyme. A crystalline complex between DP-trypsin and the soja bean inhibitor has been obtained. It, therefore, can be postulated, either that the protein inhibitors do not combine at the same site as DFP, or that these inhibitors have several points of attachment. Another trypsin inhibitor, ovomucoid, has been shown to contain one open peptide chain with alanine as N-terminal and phenylalanine as C-terminal residues [Fraenkel-Conrat & Porter (33); Pénasse *et al.* (34)]. The trypsin inhibitor present in blood plasma has been purified 50 fold by Peanasky & Laskowski (35) with a yield of 5 per cent. Its properties are different from those of the pancreatic inhibitor. It forms a stoichiometric complex with trypsin, when the salt concentration in the medium is not lower than 0.03 *M* (36).

Other inhibitors.—The inhibitory effect of organic phosphates suggests that chymotrypsin (and probably trypsin) has only one active site (esterolytic and proteolytic) which can be covered by 1 mole of inhibitor in a stoichiometric reaction. Application of the mass action law to data obtained from equilibrium dialysis experiments with chymotrypsin- α and its competitive inhibitor, α -naphthylpropionic acid, leads to a similar conclusion [Loewus & Briggs (37)]. The dissociation constant of the complex at 25°C. and pH 7.9 is $3.8 \times 10^{-3} M$, thus much higher than the aforementioned constant of the pancreatic inhibitor-trypsin system.

In a series of papers (38 to 41), Huang & Niemann have tried to substantiate their hypothesis according to which the active site of chymotrypsin- α contains three centers P_1 , P_2 , P_3 , with which trifunctional substrates $R_1CHR_2R_3$ would combine by R_1-P_1 , R_2-P_2 and R_3-P_3 specific interactions. The extent to which any compound will be bonded to the active site of the enzyme will, therefore, mainly depend upon the degree of complementarity between the molecule and the asymmetric catalytic surface and also upon the ability of both the combining molecules to alter their respective aspects to improve the closeness of fit during the complex formation. A study of various components on the kinetics of hydrolysis of nicotinyl-L-tryptophanamide and acetyl-L-tyrosinamide (trifunctional substrates) has provided examples of competitive inhibition by all three possible types of bifunctional and some monofunctional inhibitors (benzamide and indole).

When bifunctional inhibitors of the form $R(CH_2)_nR'$ (where R = benzyl or β -indolylmethyl and R' = carboxylate or carbamino) are used (38), it is possible to determine the optimum value of n to the complementary surface at the active center and also to estimate the relative importance of the various R groups in the binding process. The experimental approach to this comparative study is by measuring the dissociation constants of the complexes and calculating the standard free energy changes during their formation.

Vaslow & Doherty (42) have pursued their thermodynamic investigations on chymotrypsin complexes, already reviewed in detail last year. Schales (43) has listed a large number of new inhibitors for pepsin, trypsin, and chymotrypsin. This author expresses the view that some inhibitors act on the substrates by making them more resistant to enzymes. Siebert *et al.* (44) report an inhibition of "cathepsin" by butter yellow, and Astrup & Alkjaersig (45) propose a classification of proteolytic enzymes based on their differential inhibition by various compounds.

STRUCTURE AND ACTIVATION OF ENDOPEPTIDASES

Structure and activation of chymotrypsins and trypsin.—Comparative study of the terminal residues of the active enzymes (chymotrypsins and trypsin) and their inactive precursors (chymotrypsinogen and trypsinogen) presents a twofold interest, since it may give valuable information on the activation processes and on the peptide structure of a series of physiologically important proteins. It was already known last year that chymotrypsinogen- α is probably cyclic in structure while chymotrypsin- α contains two open chains with alanine and isoleucine as N-terminal residues [Desnuelle, Ravery & Fabre (20, 21)] and tyrosine and leucine as C-terminal residues [Gladner & Neurath (46)]. Thus, the conversion of chymotrypsinogen- α to chymotrypsin- α involves, either the opening of two cycles in chymotrypsinogen, or the cleavage of one cycle into two halves [Desnuelle & Ravery (47)]. Sorm *et al.* (23) have claimed that phenylalanine is N-terminal in chymotrypsin- α . Hydrolysates of dinitrophenylated chymotrypsin- α (DNP-chymotrypsin) contain the very stable DNP-isoleucylvaline as the N-terminal sequence of one chain; this migrates at the same rate as DNP-phenylalanine in a number of chromatographic systems. Both compounds, however, can be differentiated, either by further hydrolysis, or by paper chromatography in an aqueous phase (48). It is probably not without interest to note here that isoleucylvaline is also the N-terminal sequence of the single peptide chain of trypsin [Ravery, Fabre & Desnuelle (49)]. The question as to whether a part of the chymotrypsinogen molecule is split off during the activation, is not yet settled. Gladner & Neurath (46) have rightly pointed out that, if trypsin displays toward chymotrypsinogen the same specificity as toward synthetic substrates (see below, a discussion about trypsin specificity), one would expect to find at the C-terminal ends of chymotrypsin- α not tyrosine and leucine (as is actually the case), but lysine or arginine. The authors therefore consider that trypsin is likely to convert chymotrypsinogen- α into a primary

compound with a basic C-terminal residue. Such a compound would bear some resemblance to chymotrypsin- π , the transitory formation of which has been postulated for some years by Jacobsen (50). Chymotrypsin- π would give rise to chymotrypsins- α or δ and a basic peptide. In support of this hypothesis, it may be mentioned that different N-terminal residues are found when chymotrypsinogen- α is activated under Kunitz's or Jacobsen's conditions [Desnuelle & Røvery (47)]. The whole problem is of importance, since it may show that the formation of the well-known chymotrypsin- α is attributable to a secondary process which perhaps does not take place in the body. It is interesting to recall here the interrelationships of the various chymotrypsins, the independent existence of which has been postulated thus far by Northrop & Kunitz, Jacobsen, and Laskowski (cf. Fig. 1).

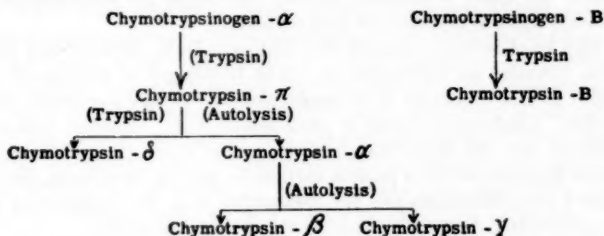


Fig. 1. Chymotrypsin interrelationships

When chymotrypsin- α is stored for a long time under conditions which favor its autolysis, new active crystals can be isolated (Kunitz's chymotrypsins- β and γ). If these enzymes are actually formed by a limited autolysis of chymotrypsin- α , a certain simplification of this last molecule would be possible without impairing its enzymatic activity. However, autolysis is not likely to take place at the N-terminal ends of the peptide chains of chymotrypsin- α since the three chymotrypsins have the same N-terminal residues in the same proportions [Røvery, Fabre & Desnuelle (51)]. A comparative study of the C-terminal residues of chymotrypsins- β and γ has not been carried out thus far.

Trypsinogen and trypsin both contain one N-terminal residue, which is valine for the zymogen and isoleucine for the enzyme [Røvery, Fabre & Desnuelle (22)]. This result suggests that both proteins contain a single open chain, the autocatalytic process consisting in the breakdown by trypsin of an isoleucine bond in the trypsinogen chain. The N-terminal end of this chain would thus be split off in the form of one or several peptides, one of which has a N-terminal valine (22). Actually, Davie & Neurath (52) have reported the isolation of a peptide which is formed during the activation and which appears to contain one valine, one lysine, and 5 (or 6) aspartic acid residues. Assuming that valine is N-terminal (as in trypsinogen) and lysine

is C-terminal (according to the specific requirements of trypsin), one can tentatively propose for this peptide the formula $\text{Val} \cdot \text{Asp}_{1(8)} \cdot \text{Lys}$. A more definite conclusion will be possible when the actual arrangement of the residues in the peptide and the N-terminal sequence of trypsinogen become known. On the other hand, Davie & Neurath (53) have found that carboxypeptidase does not split any free amino acids from either native trypsinogen or native trypsin. After being denaturated by acid, both proteins give rise to several amino acids, among which lysine is the most abundant. If both proteins actually contained the same C-terminal residue, or failed to contain any C-terminal residue, it would appear likely that the activation process did not affect the C-terminal end of the trypsinogen chain. In this case, the conversion of the zymogen into active trypsin would only imply the splitting off of a relatively short N-terminal peptide (see below for a discussion of the molecular weight of trypsinogen and trypsin).

It may be of interest to recall that last year Duke, Bier & Nord (54) had found by titration six carboxylic groups in 10,000 gm. of trypsin (that is to say about 15 free carboxyls in the molecule) which, they suggested, could be C-terminal. Linderstrøm-Lang & Max-Møller (55), however, had already pointed out that the electrometric properties of these groups are not those which can be expected from protein carboxylic groups. Obviously, the above-mentioned determinations are not compatible with the presence of such a large number of C-terminal groups in the trypsin molecule.

Some years ago, Haanes & György (56) had found that the lipotropic fraction of Bosshardt *et al.* (56a) contained trypsin and the pancreatic inhibitor of this enzyme. The fraction was activated by an enterokinase preparation. The authors, therefore, had postulated that enterokinase could overcome the action of the inhibitor. However, highly purified enterokinase is able, neither to split the crystalline trypsin-trypsin inhibitor complex, nor to attack the crystalline inhibitor itself [Mars, Peanasky & Laskowski (57)]. The activation may be ascribed to trypsinogen which is present in substantial amounts in the lipotropic fraction [Peanasky & Laskowski (57a)].

Linderstrøm-Lang & Max-Møller (55) last year briefly reviewed the work of Stahmann & Becker (58) on the addition of polymeric glycine chains to chymotrypsin. The reaction occurs under nondenaturing conditions ($\text{pH}=7.4$), the protein acting as an initiator for the polycondensation. The percentage of glycine in the enzyme can thus be raised four times without loss of activity. It would be very interesting to know at which site on the protein molecule the polymeric chains have become attached. The shift of the titration curve between pH 7 and 11 suggests that the ϵ -amino groups of lysine are likely to be the points of attachment. But more precise information could now be obtained by chemical means, especially by the fluorodinitrobenzene technique of Sanger [Becker & Stahmann (58a)].

Pepsin.—Last year, Williamson & Passmann (59) announced in a preliminary note that pepsin contains two peptide chains, one being open with leucine as N-terminal residue and the other being cyclic. This year, they

give some experimental details on the determination of the leucine N-terminal residue (60). Kern & Herriott (61) were unable to note any change in pepsin molecular weight during the treatment of the protein by mercaptans (reduction of the disulfide bridges). This observation obviously does not prove that pepsin contains only one peptide chain, since other covalent bonds may hold the chains together after the splitting of the S—S bonds and aggregation phenomena may counterbalance the primary cleavage of the molecules. However, the recent example of lysozyme, for which an analogous study has been made, suggests that pepsin actually contains one chain. Full activity is retained when one disulfide bridge (out of three) is split. The splitting of the other two brings about an inactivation which is attributed to denaturation (61).

If the molecular weights of pepsin and pepsinogen are 35,000 and 38,000, respectively, each of these molecules would contain one atom of phosphorus. This phosphorus is not essential since it can be split off by potato phosphatase without impairing pepsin activity or pepsinogen activation [Perlmann (62)]. Both proteins are also dephosphorylated by intestinal phosphatase, but pepsin is not attacked by the prostate enzyme which converts selectively the diphospho-ovalbumin A_1 into the monophospho-albumin A_2 .

The inactivation of pepsin by high intensity ultraviolet light has been investigated in great detail by McLaren *et al.* (64). The preparation, properties, and crystallization of tuna pepsin have been described by Norris & Mathies (64a).

PREPARATION AND PROPERTIES OF ENDOPEPTIDASES

Fractionation procedures.—A new and interesting technique for preparing trypsinogen has been described by Tietze (65). Autoactivation during the second crystallization is prevented by adding DFP to Kunitz's *Tg* filtrate. The reagent does not combine with trypsinogen but destroys any tryptic activity. For the first time, trypsinogen has thus been obtained in a reasonably pure form. It is stable at all pH values below 6.1. It sediments at the same rate as DP-trypsin or trypsin in acid solution. The molecular weights of trypsinogen and of trypsin monomer (see below) are, therefore, about the same. Like chymotrypsinogen, but unlike trypsin and chymotrypsin, trypsinogen does not aggregate to any appreciable extent. Enzymatic activity and aggregation capacity thus appear together.

The behavior of crystalline trypsin in the ultracentrifuge has been studied at about the same time in two laboratories (66, 67, 68). One of the publications, mainly dealing with DP-trypsin [Cunningham *et al.* (66)] was reviewed last year. The other two [Bier, Terminiello & Nord (67); Bier & Nord (68)] are in general agreement with the first. When trypsin is ultracentrifuged in an inactive state [DP-trypsin over a wide pH range (66); trypsin in acid solu-

³ Perlmann (63) now suggests that the phosphorus atom of pepsin belongs to a phospho-diester bond.

tion (66, 67, 68)] or when it is stabilized by calcium (66) its sedimentation behavior is characteristic of that of a monodisperse solute ($s_{20,w} = 2.50$ S for DP-trypsin and 2.4 S for trypsin). Assuming a partial specific volume of 0.73, the sedimentation and diffusion rates give for DP-trypsin a molecular weight of 24,000 and a dissymmetry constant $f/f_0 = 1.2$. Calculated from its phosphorus content the minimum molecular weight of DP-trypsin is 20,700. We therefore, have to deal with the weight of the monomer. On the other hand, when the ultracentrifugation is carried out under conditions in which trypsin is active and unstable, the sedimentation rate becomes dependent on pH, on temperature, and on the age of the solution, and it reveals a complex variation with protein concentration (66, 67, 68). In other words, when trypsin is active and unstable it exists in a monomer-polymer equilibrium which has led in the past to an overestimation of its true molecular weight. The reader will find in reference (66) a short but stimulating discussion on the respective aspects of trypsin and chymotrypsin- α aggregation.

Some years ago, Buchs (69) and Freudenberg (70) reported that the proteolytic activity of stomach mucosa toward edestin presents two maxima one at pH 1 to 2 (pepsin) and the other at pH 3 to 4. They attributed this last maximum to the presence of a "stomach cathepsin." This claim did not remain unchallenged in the following years, though Grassmann *et al.* (71) found that the "peptic" and the "catheptic" activities of stomach mucosa could be partly separated by paper electrophoresis. The question has been studied afresh by Heinrich (72), who compared at pH 1.8 and 3.2 the proteolytic activity of a large number of fractions separated from Difco pepsin by paper electrophoresis. No significant fractionation could be observed.

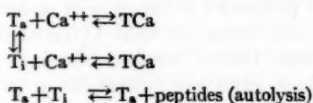
Crystalline chymotrypsinogen- α (pI ca 9) has been chromatographed on columns of the carboxylic acid resin IRC-50 [Hirs (73)]. With buffer of pH 6.0 as eluent, the protein emerged as a slightly asymmetric peak which represented 97 per cent of the ninhydrin-positive material and all the potential activity. The zymogen, as currently prepared, seems therefore to be of satisfactory purity. Furthermore, the crystals obtained by Kunitz's technique are not significantly different from the original zymogen, since pancreas extracts give an activatable peak travelling at exactly the same rate on the columns. These extracts give a second peak activated by trypsin. This peak travels with the front. It is probably Laskowski's chymotrypsinogen B (pI ca 5). The existence of this second zymogen would thus be confirmed. Trypsinogen is unstable to chromatography on IRC-50 resin.

Interactions of trypsin with divalent cations.—It is well known that calcium is not essential for trypsin activity but that it exerts on the enzyme a protective effect. This effect has been attributed thus far to a shift toward the right of the reversible equilibrium: inactive (or denatured) trypsin \rightleftharpoons active (or native) trypsin. The digestion of the inactive form by the active (autolysis) would thus be slowed down. Gorini & Felix (17a) prefer now to think that calcium simply "stabilizes" the protein trypsin against its own proteolytic activity, as is the case with other proteins (serum albumin). The main

argument in favor of the new theory is that calcium reduces the digestion of DP-trypsin by the residual tryptic activity present in this compound. Obviously, this effect cannot be attributed to an equilibrium shift.

Bier & Nord (68, 75) have found that crystalline trypsin, which is electrophoretically homogeneous at pH 5 in the absence of calcium, gives rise to two active peaks in the presence of this cation. They postulate that calcium prevents interaction of two different trypsins by combining with some carboxyl groups. These groups would, therefore, no longer be able to promote an aggregation which normally masks the electrophoretic heterogeneity. This hypothesis is in good agreement with the above-mentioned fact that calcium prevents the ultracentrifugal anomalies of active trypsin. But, before sharing the opinion expressed by Nord & Bier (75) that "trypsin has to be viewed as a heterogeneous protein consisting of two components," one has obviously to wait for some other confirmatory evidence. In the presence of calcium, the electrophoretic mobility of trypsin may be influenced by factors which have nothing to do with its fundamental chemical structure.

Trypsin seems to bind one calcium ion per mole [Green & Neurath (76)]. The following scheme is proposed by the authors for explaining the stabilizing effect of calcium, where T_a and T_i designate active trypsin and inactive trypsin respectively:



This scheme is interesting since it seems to reconcile Gorini's "old" and "new" theories. Calcium would not influence directly the equilibrium $T_a \rightleftharpoons T_i$, but it would, nevertheless, decrease the concentration of the material (T_i or DP-trypsin) which is broken down during autolysis.

Other divalent cations (Co^{++} , Cd^{++} , Mn^{++}) increase the activity of trypsin, whereas Cu^{++} , Hg^{++} , and Ag^{++} are inhibitory. The inhibitory effect is partly reversed by sequestering agents (76). Crewther (76a) has listed a great number of metals which protect trypsin against thermal denaturation at different concentrations and pHs.

As mentioned above, some proteins become more resistant to tryptic digestion in the presence of calcium ions. Manganese ions, which are known to protect lysozyme against thermal denaturation, likewise protect this protein against trypsin [Gorini, Felix & Fromageot (77)].

Gorini & Audrain (78) have studied the action of divalent cations on the trypsin-ovomucoid complex.

Rennin.—Two improved techniques for crystallizing rennin have been described (79, 80). Crystalline rennin gives three peaks on electrophoresis. The major component (75 per cent) is able to clot milk and hydrolyse proteins. The other two do not clot milk (80). We have here another example of a protein being heterogeneous after several crystallizations.

Proteolytic enzymes of various origin.—It is generally accepted that proteinase activity is largely associated with plasma, whereas enzymes acting on simple peptides are associated with the formed elements of the blood (see below, for the peptidases of erythrocytes). However, Morrison & Neurath (81) have reported the presence of three different proteinases in human erythrocytes. One is activated by reducing compounds and metals (zinc and iron), the second by metals only, whereas the third may be activated neither by reducing compounds nor by metals. The second proteinase is not inhibited by soja bean trypsin inhibitor. It does not act on the specific substrates of trypsin and chymotrypsin.

Plasminogen (profibrinolysin), the precursor of plasmin (fibrinolysin) which is found in the euglobulin fraction of blood and which lyses clots *in vitro* and *in vivo*, has been purified 400 times by taking advantage of its high resistance to acid and alkali denaturation. Starting from this purified preparation, crystalline plasminogen (not yet completely free of contaminant) has been readily obtained [Kline (81a)].

Dworschack, Koepsell & Lagoda (82) have tested for proteinase activity 491 strains of the *Aspergillus flavus-oryzae* group grown under submerged culture conditions. A highly active proteinase concentrate has been obtained from the culture medium of *Mortierella renispora* (83). Conditions have been described for optimal proteinase production in *Aspergillus terricola* cultures (84). Arginine esters and amide are split by an extracellular proteinase of *Clostridium histolyticum*. Unlike trypsin, however, the enzyme does not act on lysine methylester, and it is activatable by SH compounds [Ogle & Tytell (85)].

Determination of activity.—Although the titrimetric technique for the determination of trypsin- and chymotrypsin-esterase activity is quite satisfactory for most purposes, Parks & Plaut (86) have described a manometric technique for chymotrypsin. A neutralized solution of the enzyme is put in the side arm of a Warburg vessel, and L-phenylalanine-ethylester in bicarbonate solution is put in the main compartment. After mixing, CO₂ evolution is noted as a function of time.

Another technique consists in digesting I¹³¹-containing albumin. The undigested protein is precipitated, and the radioactivity of the supernatant is measured (87). The tryptic activity in duodenal contents has been determined by a modification of Anson & Mirsky's technique, using denatured human serum albumin as substrate (88).

ACTION OF ENDOPEPTIDASES ON THEIR SUBSTRATES

On synthetic substrates.—The specific requirements of proteolytic enzymes have been mainly studied by measuring the hydrolysis rate of various simple substrates containing different amino acid residues. The specific requirements of the enzymes, as far as the nature of the residue side chain is concerned, has been thus clearly defined. It is of an obvious interest to study now the degradation of synthetic polymers containing only one kind of

amino acid residues. In this way, one can investigate conveniently the influence of the position of the bonds on the rate of their proteolysis by endopeptidases. Polylysine is readily attacked by crystalline trypsin. Using chromatographic techniques, Waley & Watson (89) have shown that the exo bonds (next to the N-terminal or C-terminal residues) are not split by trypsin but those near the end are preferentially split, so that the main products of the degradation are lower lysine peptides. Furthermore, the well-known inhibitory effect of charged groups on endopeptidase activity has been confirmed. Pentalysine is rapidly degraded to di- and trilylsine. Tetralysine gives rise more slowly to dilysine. Trilylsine and dilysine are not attacked by trypsin. Finally, the chemical identity of all the residues along the chain allows a comparison to be made between polylysine degradation by trypsin and amylose degradation by α and β amylases. In this interesting paper the authors also describe a new potentiometric technique for the determination of the total number of peptide bonds which are broken down during hydrolysis.

Like other tyrosine amides, the amide of dibenzoyl glyceryl-L-tyrosine is a good substrate for chymotrypsin- α [Doherty (90)]. Hogness & Niemann (91) have investigated the kinetics of acetyl-L-tyrosinhydroxamide hydrolysis by the same enzyme.

On proteins and peptides.—Two papers are devoted this year to the conversion of bovine fibrinogen to fibrin, a process which is catalysed by thrombin. Lorand & Middlebrook (92) have analysed the peptide material, named by them fibrino peptide, which is split off during the conversion. Chemical analysis would indicate a minimum molecular weight of about 2,200. The molecular weight, determined by the amount of N-terminal glutamic acid reactive with dinitrofluorobenzene, is 8,000. However, fibrino peptide is probably not homogeneous. The appearance of four new glycine N-terminal residues on fibrin suggests the formation of four peptides, among which only two can have an N-terminal glutamic acid (corresponding to the two N-terminal glutamic acid residues lost by fibrinogen during the clotting). Actually, two ninhydrin-positive bands are formed when fibrino peptide is submitted to paper electrophoresis at pH 4.15. One of these bands contains N-terminal glutamic acid and no lysine. The other contains no reactive N-terminal residues but all the lysine [Bettelheim & Bailey (93)]. The detailed study of this peptide material is somewhat complicated by the fact that cyclisation apparently occurs when solutions are taken to dryness or lyophilized.

When used at very low concentration [Steinberg (94)], the *Bacillus subtilis* protease seems to "open" the ovalbumin molecule instead of splitting off the well-known hexapeptide analyzed by Ottesen & Wollenberger (95). A new, plakalbumin-like, protein is formed which contains an alanine residue available to carboxypeptidase (thus apparently C-terminal). The previous claim (96) of alanine being C-terminal in ovalbumin itself, has been withdrawn (94). Chymotrypsinogen and ovalbumin seem to be devoid of any N- and C-terminal residues. It is therefore likely that both proteins are

cyclic. The fact that ovalbumin proteolysis depends on the amount of enzyme employed, is very interesting. It would suggest that endopeptidase specificity may be more restricted when low concentrations of enzyme or unfavorable conditions (pH, temperature, partial inhibition) are used.

Drucker, Hainsworth & Smith (97) have reinvestigated the action of proteolytic enzymes on silk fibroin solubilized by cupri-ethylenediamine. The insoluble fraction formed during the digestion with pancreatin contained mostly glycine, alanine, and serine in a molar ratio 3:2:1 and a little tyrosine. It contained more tyrosine when chymotrypsin was used, and all the amino acids of fibroin when trypsin was used. If it be assumed that no rearrangement occurs during the proteolysis and that the degradation proceeds as required by the specific requirements of the enzyme, these results would throw considerable light on the structure of fibroin and, in a more general way, would be a good example of the usefulness of enzymic digestion for the investigation of protein structure. Another example is given by Steinberg & Anfinsen's beautiful work (98). Peptic hydrolysates of ovalbumin in which labeled alanine was incorporated *in vitro* and *in vivo*, have been chromatographed. Peptides with alanine fractions differing in specific activity by as much as 400 per cent have been obtained. Thus a stimulating discussion can be made concerning the respective role played during protein synthesis by the template or the stepwise mechanism.

When incubated with trypsin, myosin solutions undergo a considerable fall in viscosity whereas the nonprotein nitrogen rises slowly. By ultracentrifugal studies [Mihalyi & Szent-Györgyi (99)], it appears that the protein is very quickly broken down by trypsin to two kinds of particles, the first sedimenting more slowly than native myosin and the second, more rapidly. This last result is surprising. It is explained by assuming that the second component is less asymmetric than myosin. Both components, which still possess a relatively high molecular weight, are further degraded at a slow rate. Contrary to Mihalyi & Szent-Györgyi's statement, it is difficult, however, to think that myosin degradation by trypsin belongs to the all or none type. According to Tiselius' theory, proteins undergo an all or none degradation when the molecules are broken down one after the other. Then, intact molecules disappear rather slowly, and the reaction mixture contains intact molecules (in decreasing proportions) and smaller peptides of roughly constant molecular weight (in increasing proportions). The rapid phase of myosin degradation follows first order kinetics [Mihalyi (100)], and the adenosinetriphosphatase activity, as well as the actin-binding capacity, are associated with the faster sedimenting component. Since no net loss of activity occurs, it is postulated that the slower sedimenting component split off by trypsin represents an "inactive" part of the myosin molecule [Mihalyi & Szent-Györgyi (101)]. It may be of interest to mention here that the contractility and excitability of rat heart muscle are much depressed by crystalline trypsin [Green (102)].

Elastase dissolves elastin, converting it from the fibrous to the globular state and liberating free amino and carboxyl groups (103).

Yasnoff & Bull (104) have reported that an insoluble but not denatured complex between pepsin and egg albumin is formed at pH 4.0. The optimal molar ratio is about 1:1. The precipitate dissolves when an excess of pepsin or albumin is added or when the ionic concentration is increased. It does not seem likely that this complex results from a specific enzyme-substrate interaction, since its association constant is ten times as high as the constant calculated by the Michaelis-Menten treatment of the proteolysis at pH 2. Further, the association seems to be controlled by an unspecific electrostatic attraction between two proteins of opposite net charge. Nevertheless, the phenomenon is quite interesting in the light of another work by Mazia & Hayashi (105) in which fibers were prepared by compressing a mixed monolayer of albumin and pepsin at pH 4.0. Pepsin, which is probably in a partly unfolded state, was still active, and the fibers digested themselves at pH 1.5. This may be a far-reaching observation for all enzymatic reactions taking place in rigidly organized systems.

In 1939, Behrens & Bergmann had noted that some peptides are not attacked by papain unless other peptides are simultaneously present. They supposed that this effect (co-factor effect) was a result of a condensation of the peptide substrate, A.B, with the co-factor, C.D. The enzyme, unable to split the bond A—B in the peptide A.B, would then be able to split this bond in the condensation product A.B.C.D. This problem has been reinvestigated by Johnson & Herriott (106). They could confirm that glycnamide and leucylglycine are not hydrolysed by activated papain unless certain other peptides, which are not attacked during the process and need not contain specific amino acid residues, are also present. Cofactors of high activity are formed during the papainolytic digestion of horse-serum albumin. This latter observation would suggest that co-factor action may occur during proteolysis and that papain specificity may be influenced by the composition of the reaction mixture. However, the authors could not detect any of the condensation products required by the hypothesis of Behrens & Bergmann. These products, therefore, are not formed or are broken down very fast.

Trypsin has been reported to convert profibrinolysin of dog serum into active fibrinolysin (107, 108). The fibrin-plate method, originally described for the estimation of fibrinolytic activity, also has been found convenient for measuring the activity of various proteolytic enzymes, including trypsin, chymotrypsin, papain, ficin, and some bacterial proteases (108 to 111).

The specificity of trypsin action toward the arginyl and lysyl bonds of synthetic substrates is well known. When acting on proteins or large polypeptides, the enzyme seems to display the same, narrowly restricted specificity. It splits, for instance, an arginyl-glycine bond in insulin B-chains (112), no bonds in insulin A-chains (which are devoid of any basic residues), an arginyl-glycinamide bond in bovine vasopressin (113, 114), and probably, a

lysyl-isoleucine bond in trypsinogen (52, 115). On the other hand, the only amino acid liberated from horse globin by crystalline trypsin is lysine (116). This specificity, which may explain why trypsin is unable to attack some proteins in the native state, is highly interesting for the study of protein structure. The specificity of chymotrypsin and pepsin seems to be less clear-cut.

Lens & Evertzen (117) for the first time have used the starch columns of Stein & Moore for the investigation of enzymatic digests. The results are encouraging and the authors emphasize the usefulness of the technique for the detailed study of the tryptones used in microbiological culture media. Grassmann & Deffner (118) have reported that phenol-butanol-acetic acid mixtures are good solvents for the fractionation on paper of the higher peptides formed during proteolysis.

Synthesis of peptide bonds.—The reversal of proteolytic action (synthesis of peptide bonds by proteolytic enzymes) presents a twofold interest. On the one hand, it may represent one way by which proteins are synthesized *in vivo*. On the other hand, it may constitute a danger when enzymic digests are prepared for the study of protein structure. Several mechanisms have been proposed for the supply of the necessary energy during synthesis (stores of ester, amide, and peptide bonds which are utilized by transpeptidation; energy-rich phosphate bonds, etc.). But the question whether or not the proteolytic enzymes with which we are acquainted so far, actually play a role in protein synthesis *in vivo*, is not yet settled. In a comprehensive, clear, and well-documented review, Campbell & Work (119) have listed and discussed various possibilities. Loftfield *et al.* (120) have pointed out that radioactive α -aminobutyric acid is incorporated into proteins only to a very slight extent when incubated with rat liver slices. However, the acid penetrates freely into the cells, and liver proteolytic enzymes are able to split lower aminobutyryl peptides as well as lower alanyl peptides. These facts are taken by the authors as ruling out any active participation of the proteolytic enzymes in protein synthesis, although the choice between "right" and "wrong" amino acids may be made by enzymes synthesizing larger peptides. Janssen, Winitz & Fox (121) have reinvestigated the reactions occurring when ficin, papain, and chymotrypsin act on a mixture of benzoyl-phenylalanine and glycylalanilide. Chymotrypsin gives rise to benzoylphenylalanyl-glycylalanilide by a coupling reaction. Ficin and papain form benzoylphenylalaninyl-glycylalanilide and benzoylphenylalanyl-glycylglycylalanilide, indicating that transamidation and transpeptidation reactions also occur with these enzymes.

Fruton *et al.* (121a) have pursued their investigations on the polymerisation of dipeptide amides in the presence of cathepsin C. With glycyl-L-phenylalaninamide, polymeric chains (mean length: 8 to 10 residues) are formed. This polymerisation seems to involve successive transamidations, glycyl-L-phenylalanyl units being added each time at the amino end of the growing chain. Similar polymerisations have been noted with other sub-

strates of cathepsin C (glycyl-L-tyrosinamide, L-alanyl-L-phenylalaninamide, L-alanyl-L-tyrosinamide).

EXOPEPTIDASES

Carboxypeptidase.—Pancreatic crystalline carboxypeptidase of bovine origin has only one reactive N-terminal residue, which is asparagine [Thompson (122)]. As is often the case with aspartyl N-terminal residues, this one is fairly unstable during the condensation with fluorodinitrobenzene. Serine is the next residue. As a result of the well-known lability of serine bonds, no other residue has thus far been identified.

Ronwin (123) has found that hippuryl derivatives, particularly hippuryl-leucine, are most potent substrates for pancreatic carboxypeptidase. In the same paper, the author develops a new theory of enzyme action (dipositive-bond theory) which is in general accord with the results obtained with carboxypeptidase and various synthetic substrates. The details of this theory will be found in the original. "Enterokinase-activated carboxypeptidase" has been reported to hydrolyse α -bromoisobutyryl-*m*- or *p*-aminobenzoic acids, but not the ortho compound (124). The immunochemical reactions of carboxypeptidase have been investigated by Smith *et al.* (125).

Carboxypeptidase activity does not seem to be restricted to the pancreas. It is apparently distributed throughout mammalian tissues. Previous failure to detect this activity is probably attributable to the existence of a natural cellular inhibitor which can be separated by high speed centrifugation [Feinstein & Ballin (126)]. According to Ballin & Feinstein (127), the various carboxypeptidases appear in two forms, one activated and the other inhibited by cysteine.

Pancreatic carboxypeptidase has been used for determining the C-terminal residues of a number of proteins (1). The limitations of the technique appear more and more clearly. Firstly, the inability of carboxypeptidase to split off a free amino acid from a protein, is no proof that this protein does not contain any C-terminal residue. The residue may not fulfill the specific requirements of the enzyme or it may be sterically unavailable. Secondly, let us suppose a protein with one open chain, in which the C-terminal residue is "wrong" for the enzyme and the adjacent residue is "right." The splitting of the terminal residue is rate-limiting, the second residue appears almost simultaneously, and one may wrongly conclude that the protein has two open chains. Thirdly, when the protein contains several open chains, the penultimate residue of the first chain may be freed more rapidly than the terminal residue of the second. Finally, carboxypeptidase may be contaminated by some proteases which, in certain cases, considerably alter its action (94). This last possibility seems to be largely ruled out when diisopropylfluorophosphate (to which carboxypeptidase is fortunately not sensitive) is used. However, the present lack of a simple and entirely secure chemical technique for determining protein C-terminal residues has led to an extensive use of

carboxypeptidase as an analytical tool. The C-terminal residues of nine proteins have already been investigated in this way (1, 128). Dinitrophenylated proteins prepared by Sanger's method are also attacked by the enzyme (129).

Other exopeptidases.—Some authors have expressed the view that such restricted designations as "leucine" amino peptidase may be ambiguous, and they have suggested that the problem of substrate specificity can be solved only through progress in the purification of individual peptidases. In this connection, it is interesting to note that no amino peptidases have been crystallized so far, despite the stimulating example of pancreatic carboxypeptidase which was crystallized by Anson 15 years ago.

Smith, Spackman & Polglase (130) have reinvestigated the specific requirements of a leucine amino peptidase preparation from swine intestinal mucosa. The nature of the N-terminal residue of the substrate is of obvious importance. The hydrolysis rate of certain amides used as substrate was slowed down by 90 per cent, when L-leucine was replaced by L-valine or L-isoleucine. The chemical nature of the C-terminal residue was found to be much less important, but its stereochemical configuration must be L. Otherwise, hydrolysis did not occur, or it was very slow. The effect of various metals on leucine amino peptidase has been studied (131).

Robinson, Birnbaum & Greenstein (132) have purified an amino peptidase from kidney cell particulates which, unlike the soluble enzyme of the same organ, was able to split glycyl-D-alanine and glycyldehydroalanine. The activity of the freshly prepared enzyme was 600 times as high as that of kidney homogenates. It increased 2 to 3 fold during a 2 to 3 month storage. Thirty per cent by weight of the preparation appeared to be soluble in lipid solvents.

Amino peptidases from calf thymus, rabbit skeletal muscle, and human synovial fluid are inhibited by a group of compounds known to possess antihistaminic and anticholinesterase activities (133). The hydrolysis of some peptides by surviving rat tissue diaphragm (134) and beef thyroid (135) has been reported.

The existence of enzymes acting specifically on prolyl peptides (prolinases) has been postulated for a long time. This year, Davis & Smith (136, 137) have reported the purification of an "iminodipeptidase" from swine kidney and the crystallisation of a "prolidase" from the same organ. "Iminodipeptidases" are defined as enzymes acting on dipeptides which possess the free imino group of proline or hydroxyproline. The specificity of the renal enzyme is very narrowly restricted, since it has no action on L-prolyl-amide, L-prolylglycylglycine, or glycyl-L-prolylglycine. "Prolidase," on the other hand, is defined as an enzyme which specifically hydrolyzes the peptide bonds involving the imino nitrogen of proline and hydroxyproline. In specific substrates for imino peptidase, proline is therefore N-terminal, and it is C-terminal in specific substrates for prolidase. The crystalline prolidase from swine kidney appears to be at the same time a metallo and a sulfhydryl enzyme, since it is activated by Mn^{++} or mercaptans and inhibited by mer-

captide-forming reagents. Another prolidase, activable by Mn^{++} , is also found in horse erythrocytes (138). According to Smith's nomenclature, a prolineless mutant of *Escherichia coli* appears to contain an iminodipeptidase and a prolidase, since it hydrolyzes prolylglycine, glycylproline, and also both peptide bonds of glycylprolylglycine in the presence of Mn^{++} and cysteine [Stone (139)].

As far as exopeptidases of the blood are concerned, it has been shown that hemolyzed erythrocytes of man and other species and nucleated avian erythrocytes hydrolyze L-leucine amide, glycylglycine, triglycine, and glycyl-L-proline. Hence, amino peptidase, dipeptidase, tripeptidase, and prolidase activities have been attributed to these cells [Adams, McFadden & Smith (140)]. In addition to erythrocyte prolidase, already referred to, erythrocyte tripeptidase has been investigated in detail [Adams, Davis & Smith (141)].

It has been observed that the kinetics of glycylglycylglycine hydrolysis by human serum or red blood cells fits closely with the curve derived from the integrated Michaelis-Menten equation [Fleisher (142)]. A number of peptides have been acted upon by human, guinea pig, and rabbit serum [Hanson & Wenzel (143)].

Fodor, Miller & Waelsch (144) have found that the enzymatic cleavage of glutathione (to glutamic acid and cysteinylglycine) by purified kidney, liver, and brain preparations, is accelerated by various amino acids and peptides which probably act as acceptors for the γ -glutamyl radical. The most potent activators are glycylglycine and glycyl-L-alanine. Glutamine, γ -ethylglutamate, methionine, and ethionine are also active. The other amino acids, including glycine, have a lower activity. D-Amino acids and N-substituted amino acids are without action.

Greenstein *et al.* (145 to 152) have continued their extensive investigations on the stereochemical resolution of N-acylamino acids by acylases.

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CHEMISTRY OF THE CARBOHYDRATES^{1,2}

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Rapid expansion of the carbohydrate literature makes it very difficult to render a comprehensive conspectus of all developments during the past year. Consequently the present discussion is limited to those aspects of carbohydrate chemistry which concern techniques, oligosaccharides, and polysaccharides. The last-mentioned is given particular attention because it has been some time since polysaccharides were discussed at length in this series. Several extensive reviews of polysaccharides appeared recently and should be helpful to persons interested in those areas. A new edition of *Wood Chemistry* (1) lucidly presents the modern views concerning the properties and chemistry of various woods and their components. The first volume of the third edition of *Starch and Its Derivatives* (2) will be of value to chemists interested in this field. In the book *Polysaccharide Chemistry* (3) the entire field of polysaccharide chemistry is brought together and all known polysaccharides are classified and thoroughly described. Smaller reviews are concerned with the properties of pectin (4) and the chemistry of agar (5).

A series of carbohydrate nomenclature rules have been formulated by the joint action of the American and British Committees (6). Since the rules are the culmination of many years' work, they represent substantial advances, and it is hoped that authors will follow these rules to facilitate uniformity in the literature and a clearer understanding of presentations.

TECHNIQUES

Electrophoretic procedures have been added to the elegant paper chromatographic methods for the separation of sugars and their derivatives. In almost all instances borate buffers are employed, frequently at pH 10. Most simple sugars and oligosaccharides are quickly and easily separated (7 to 11). Structural details may be suggested by the rate at which the borate complexes migrate. Instead of placing the paper in a horizontal position it may be held vertically and electrophoretic separation effected while downward flow is in progress (12). Horizontal paper electrophoresis can be employed for the rapid separation of hyaluronic acid, chondroitin sulfuric acid, and heparin (13). Chondroitin and hyaluronic acid can also be separated by electrophoresis in a slab of "Hyflo Super-Cel" (14). It is possible to separate and determine P³²-labeled hexose phosphate esters in mixtures by electrophoresis on paper. Analytical use of this technique is helpful in following many enzymic reactions and in obtaining rapid quantitative analysis of complex mixtures (15).

¹ The survey of the literature pertaining to this review was completed in October, 1953.

² The following abbreviation is used in this chapter: D.P. for degrees of polymerization.

A chromatographic procedure is described for the separation of aldehydes and ketones on filter paper pretreated with sodium bisulphite solution. The aldehydes, being the more reactive towards this reagent, tend to be retarded during the development of the chromatogram. Information regarding the functional groups in unknown compounds may be obtained by the use of such treated papers (16).

Further advances have been announced in techniques for the separation on a preparative scale, of mixtures of the more simple carbohydrates. The ion-exchange-borate buffer method developed for monosaccharide mixtures by Khym & Zill (17) is extended to cover mixtures of sugar alcohols (18), sugar phosphates (19), and a mixture of L-arabinose and D-galactose with D-glucuronic and D-galacturonic acids (20). D-Glucosamine and D-galactosamine are separated and quantitatively determined by Gardell (21). The method depends upon elution of the amino sugars with 0.3*N* hydrochloric acid from a column of Dowex 50 ion-exchange resin. A method for the distinction between D-glucosamine and D-galactosamine lies in their oxidation by ninhydrin to D-arabinose and D-lyxose respectively, followed by the separation and identification of these pentoses by paper chromatography (22). Gardell elaborates further a method in which a mixture of monosaccharides is separated and quantitatively determined. Sugars are eluted from a starch column with a butanol-propanol-water solvent system and directly determined in the eluant fractions by a color reaction with aniline trichloroacetate (23).

Mould & Syngé (24) obtained separation of a series of α -1 \rightarrow 4 linked polysaccharides using electrokinetic ultrafiltration through gel membranes of varying porosities. Membranes with a small pore radius give a separation of the smaller molecules having D.P.² from 35 to 75. Larger molecules, D.P. 100 to 250, are separated by membranes with a greater pore size.

Chromatographic procedures, whether on paper or columns, are to be recommended as important aids in the determination of polysaccharide structure. The nature of the glycosidic linkages in polysaccharides can be deduced from the oligosaccharides produced on partial hydrolysis. In following this route of structure determination, workers must keep several factors in mind to avoid the assignment of erroneous constitutions to the polymer under investigation. Hydrolysis of polysaccharides whether by acid or by enzymes is an equilibrium reaction wherein monosaccharides are condensed to form new oligosaccharides which are not necessarily those produced as fragments from the polysaccharide. The time necessary for polysaccharide hydrolysis, in general, is far less than that necessary to attain equilibrium. Nonetheless, small traces of equilibrium products may be formed. Since chromatographic methods are so sensitive, traces of compounds can be detected and often isolated. A paper concerned with the effects of heat on a solution of D-glucose and D-glucose polymers draws particular attention to this fact of hydrolysis equilibrium (25).

It is also important to note that interconversion of aldose and ketose

sugars can be effected by alkaline impurities in the paper of a chromatogram (26). D-Fructose, D-glucose, L-rhamnose and D-xylose are partly converted when solutions are dried on chromatogram paper at 110°C. No conversion is observed on drying at room temperature. Raffinose, sucrose, D-galactose, D-mannose, L-sorbose, D-glucosamine hydrochloride, ascorbic acid, and inositol are not affected. Under this condition D-galacturonic acid is partly converted to the lactone. The presence of sodium and potassium can be demonstrated in water washings of chromatography paper. Although appreciable decomposition of sugars takes place on heating at 110°C., good recoveries are obtained if the initial spots of sugar solution are dried at room temperature.

A rapid procedure for the quantitative determination of D-glucose in a mixture with other sugars involves its selective oxidation with glucose dehydrogenase in the presence of air to produce D-gluconic acid which is titrated with standard alkali (27).

Recent investigations show that the oxidation of carbohydrates with glycol-splitting reagents is not restricted to "Malapradian type" reactions (28). Periodate reacts with 2,3,4,6-tetra-O-methyl-D-glucose, tri-O-methyl-D-glucopyranoses and other methoxy compounds. The reactions of the methylated carbohydrate derivatives take place readily at pH 7.5 but only slowly at pH 5. These reactions are not dependent on hydroxyl groups, but the rate of reaction increases with the number of methoxyl groups.

As a result of an investigation into the effect of daylight on periodate oxidation, it is reported (29) that formic acid, formaldehyde, and other non-glycolic simple organic substances are oxidized to carbon dioxide and water. Similar reactions take place in the dark, but much more slowly. These results are of practical importance since formic acid and formaldehyde are normally considered to be end-products in the periodate oxidation of carbohydrates, and they are determined as such in structural investigations.

Light accelerates the periodate oxidation of methyl β -D-glucoside, methyl β -cellobioside, and cellulose (30). It is doubtful if the initial Malapradian reaction is affected, but the rates of the subsequent over-oxidation are increased greatly. Over-oxidation does take place in the dark, and ultimately the same results are obtained as in light-accelerated reactions. If sufficient oxidant is used, reaction proceeds until the organic matter has been oxidized to carbon dioxide and water. A possible mechanism of the over-oxidation of cellulose is discussed.

Neumüller & Vasseur (31) studied the influence of pH, between strong acidity and neutrality, on the periodate oxidation of some carbohydrates. In weakly acidic solutions the rapid Malapradian reaction is followed by a more or less rapid over-oxidation, the rate of which is minimal in the region of pH 3 to 4. All the sugars tested consumed large amounts of periodate and were broken down completely by oxidation in a neutral medium. The mechanism of acidic over-oxidation is discussed.

1,6-Anhydro- β -D-glucofuranose (32), 1,6-anhydro- α -D-galactofuranose (33)

and D-glucaric-1,4-lactone (34) contain glycol groupings, but they are resistant to oxidation by periodic acid, sodium periodate, and lead tetraacetate.

OLIGOSACCHARIDES

Through use of established chromatographic methods (35, 36), it is now possible to separate oligosaccharides from each other in workable quantities. Numerous oligosaccharides are being obtained from partial depolymerization of polysaccharides or by synthesis. Those produced by transglycosidation reactions were thoroughly summarized in the 1953 *Annual Review of Biochemistry*. There is a particularly wide interest in producing new oligosaccharides through *in vitro* enzyme action. It is unfortunate that this enthusiastic expansion of the oligosaccharide area has in some cases brought more confusion than enlightenment by: (a) identification of oligosaccharides only by paper chromatograms and (b) the desire, on the part of some workers, to confer trivial names on new sugars. Chromatographic mobilities and colors produced by spray reagents cannot be used as positive identification of compounds or of structures. For unequivocal identification, only isolation and characterization of crystalline compounds is acceptable. The number of oligosaccharides theoretically producible is astronomical and many hundreds may be isolated within the next few years. Consequently, chemists should not be burdened with the need for learning trivial names, particularly for the great majority of oligosaccharides which have no outstanding chemical or practical importance.

A crystalline trisaccharide (kestose) (37), produced during the action of yeast invertase on sucrose, has been isolated (38) by cellulose chromatography and shown by methylation methods to be O- β -D-fructofuranosyl-(2 \rightarrow 6)-O- β -D-fructofuranosyl α -D-glucopyranoside (39). A similar trisaccharide can be extracted from tubers of the Jerusalem artichoke (40).

The principal trisaccharide produced by honey invertase on sucrose is O- α -D-glucopyranosyl-(1 \rightarrow 4)-O- α -D-glucopyranosyl β -D-fructofuranoside; a structure deduced from the observation that on partial hydrolysis there is obtained both maltose and sucrose (41). A similar trisaccharide composed of two D-glucose units and one D-fructose unit, can be isolated by charcoal chromatography from the honeydew of the citrus mealy bug *Pseudococcus citri* when feeding on the sap of potato sprouts (42). Presumably this trisaccharide arises as a natural product in the digestive systems of many insects. Also there is chromatographic evidence for its presence in honeydews of cottony maple scale, *Pulvinaria vitis*, and in the spirea aphid, *Aphis spiricola*.

A lactase preparation from *Saccharomyces fragilis* acts on lactose to bring about hydrolysis, but with the simultaneous formation of some ten oligosaccharides, most of which can be separated by charcoal chromatography (43). Although the oligosaccharides are incompletely identified, hydrolysis shows that some are composed of D-galactose units only while some contain both D-galactose and D-glucose units.

By alcohol extraction of wheat flour there are obtained several oligosaccharides, one of which hydrolyzes to D-fructose and raffinose (44). The same series of oligosaccharides is obtained by partial acid hydrolysis of potato amylose (45). There is some evidence to indicate that maltotriose is unfermentable by yeast (46). An interesting polymer-homologous series of oligosaccharides extending thus far to the tetrasaccharide, is readily obtained by partial enzymatic hydrolysis of pectic acid (47). The monomer units are D-galacturonic acid which are presumably linked as in pectic acid; that is to say, in the α -1 \rightarrow 4 configuration.

Numerous other oligosaccharides have been prepared and additional information obtained on those already known. Inulobiose is obtained from the hydrolysis of inulin and is probably 1-O- β -D-fructofuranosyl-D-fructose (48). Cellobiose has been isolated and definitely identified as an intermediate in the enzymatic hydrolysis of cellulose (49). Stachyose, although long known from such sources as *Stachys tuberosa*, has had several structures suggested for it. However, its structure is now definitely shown to be O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- α -D-glucopyranosyl β -D-fructofuranoside (50). A trisaccharide (planteose) isolated (51) from the seeds of *Plantago major* and *P. ovata* is shown to be O- α -D-galactopyranosyl-(1 \rightarrow 6)-O- β -D-fructofuranosyl α -D-glucopyranoside (52).

A disaccharide which is shown to be 3-O- β -L-arabopyranosyl-L-arabinose has been isolated from a dilute acid hydrolyzate of larch ϵ -galactan (53). A nonreducing trisaccharide can be isolated from the products of action of "Takadiastase" on sucrose. Methylation of the trisaccharide and analysis of the partially substituted sugars produced on hydrolysis shows the structure to be O- β -D-fructofuranosyl-(2 \rightarrow 1)-O- β -D-fructofuranosyl α -D-glucopyranoside. It appears to be formed by enzymatic transfer of a β -D-fructofuranosyl radical to sucrose (54). Some recent chemical syntheses of disaccharides may be noted. Sucrose can be synthesized by reaction of 3,4,6-tri-O-acetyl-1,2-anhydro- α -D-glucopyranose with 1,3,4,6-tetra-O-acetyl-D-fructofuranose in a sealed tube at 100°C. for 104 hr. Chromatographic separation of the products of the synthesis gave a 5.5 per cent yield of sucrose octaacetate (55). Octaacetyl- β -D-maltose is synthesized by treatment of the above anhydro-sugar with 1,2,3,6-tetra-O-acetyl- β -D-glucose at 120°C. for 13 hr. (56). Laminaribiose (3-O- β -D-glucopyranosyl-D-glucose) is the product of reaction of 1,2:5,6-di-O-isopropylidene-D-glucose with tetra-O-acetyl- β -D-glucopyranosyl bromide (57).

POLYSACCHARIDES

Hemicelluloses.—One of the major problems confronting the polysaccharide chemist is that of extraction of his material prior to its structural investigation. It is essential that the molecular structure of the isolated polysaccharide be changed as little as possible by the extraction process. To obtain hemicelluloses, alkaline extraction of native plant material has been widely employed but it is now customary to start with the specially delignified

pulp, holocellulose. Two delignification methods are in common use; the chlorite and the chlorine-ethanolamine methods. The former, carried out at a pH near neutrality is said to not affect appreciably the polysaccharides of the sample. However, the material after treatment may contain up to 3-5 per cent lignin. If attempts are made to remove this residual lignin, longer reaction times must be employed with concomitant loss of polysaccharides (58 to 61). The latter method, as modified by Timell & Jahn (62), is said to result in no loss or degradation of paper birch polysaccharides when the lignin content is reduced to 0.2 to 0.4 per cent. The soluble polysaccharides are obtained by extraction of this holocellulose with water or aqueous alkali, from which solution they are precipitated by addition of ethanol.

Wheat straw and wheat straw holocellulose can be dispersed in cupri-ethylenediamine solutions and a fractionation obtained by graded precipitation with acid and alcohol. A comparison of the hemicelluloses obtained from the two starting materials shows that the acid chlorite used in the preparation of the holocellulose causes very little hydrolytic action (63). If the investigator is interested in structures containing labile linkages, however, such as that of L-arabofuranoside, it is possible that he would need to employ extracts of native material.

Swelling treatment with liquid ammonia can be used to increase the accessibility of the holocellulose to extraction solvents. No marked drop is observed in the degree of polymerization of the residual celluloses after liquid ammonia treatment and hemicellulose extraction, as compared with a straightforward aqueous and alkaline hemicellulose extraction of birch and spruce holocellulose (64, 65, 66).

The resistance to extraction of some polysaccharides is no doubt attributable to the fact that they are held tightly by numerous secondary forces or so overlaid or entangled with other types of molecules that they are not able to disperse in the extracting solvent. The swelling in liquid ammonia may serve to expand the holocellulose structure resulting in an increase in the weight of water-extractable polysaccharides from 3 per cent to 20 per cent. It is possible, however, that the hemicelluloses, cellulose, and lignin are linked to some extent covalently. Evidence for this concept of plant structure is found in the isolation and characterization of oligosaccharides built up from D-glucose and other monosaccharide residues which might indicate a linkage between cellulose and hemicellulose (see *Cellulose*). Several workers claim to have found evidence for the existence of a carbohydrate-lignin bond (67, 68). A review of the carbohydrate-lignin bond literature has been published recently (69).

It is pointed out that some hydrolysis of cell-wall constituents might take place during the extraction of plant materials with hot water. Thompson, Becher & Wise (70) find a hot water extract of previously cold water-extracted Douglas fir to have a pH of 3. The composition of the hydrolyzate of this hot water extract differs from that of the cold water extract and approaches that of a typical hemicellulose preparation. This possibility of

hydrolysis during hot water treatment has been mentioned previously by Green & Leaf (71) and also by Browning (72). It is of interest to note that treatment of hardwoods with water at 140 to 150°C. (prehydrolysis) prior to sulfate cooking, facilitates the production of pulps of lower pentosan content than is possible without prehydrolysis.

A new avenue of investigation may be opened by the discovery (73) that synthetic glucans and glucans of plant and animal origin give specific precipitates in the cold with antibodies developed in the horse to pneumococcal Types II, IX, XII, XX, and XXII. Saliva destroys the serological activity of corn and oyster glycogens, the only ones so tested. Amylopectin and a limit dextrin give smaller precipitates. It is considered that certain valid deductions are possible as to the relation between the chemical constitution of glucans and their immunological specificity.

Hemicellulose preparations are often polysaccharide mixtures, and their separation is proving a problem of some magnitude. Graded addition of ethanol to aqueous or dilute alkaline solutions of hemicelluloses, or addition of petroleum ether to the solution of the acetate in chloroform, are two methods that have been tried in attempted fractionations of hemicellulose preparations, but so far without convincing success (63, 74).

Graded extraction of wheat straw holocellulose, with cold and hot water followed by increasing strengths of alkali results in partial fractionation of wheat straw hemicelluloses. That material extracted by hot water can be purified by petroleum ether precipitation of the acetate from chloroform solution, to yield a product with a constant ratio between xylose, arabinose, and hexuronic acid. Further investigations of this hemicellulose suggest that it consists of a chain of approximately 30 D-xylose units linked β -1 \rightarrow 4 and to this chain are attached by means of 1 \rightarrow 3 links, five L-arabinose units and three D-glucuronic acid units as side groups (75, 76).

It is possible to approach the structural characterization of hemicelluloses by an examination of the oligosaccharide fragments produced on partial hydrolysis. Bishop has isolated and identified an aldobiouronic acid obtained from a wheat straw hemicellulose. This compound has been shown by degradation and synthesis to be 3-O-(α -D-glucopyranosyluronic acid)-D-xylose (77).

That portion of the alkali soluble hemicelluloses of corn cob which remains in solution on neutralization of the extract is not well-characterized and is termed hemicellulose-B. This fraction contains a higher percentage of uronic acids than the less soluble hemicellulose-A. Chain fragments are obtained by partial hydrolysis, and three of these are identified as 2-O-(4-O-methyl- α -D-glucopyranosyluronic acid)-D-xylose (74), 2-O-(α -D-glucopyranosyluronic acid)-D-xylose, and 4-O-(α -D-glucopyranosyluronic acid)-D-xylose (78).

The chemical constitution of wood hemicelluloses has been little investigated. Jones & Wise (79) find L-rhamnose, L-arabinose, D-xylose, D-galactose, xylobiose, xylotriose, 4-O-methyl-D-glucuronic acid, and several oligosac-

charides containing uronic acids in a hydrolyzate of extractive-free aspen wood. The same authors later identify 2-O-(4-O-methyl- α -D-glucopyranosyl-uronic acid)-D-xylose after fractionation of the aldobiouronic acids produced on hydrolysis of aspen sawdust. Galactose and rhamnose can be identified in a birch wood hydrolyzate (80). The need to separate hemicellulose polysaccharides is reflected in a paper which concerns an arabinose-rich fraction of Esparto hemicellulose (82). Three different methylated derivatives of D-galactose, two of L-arabinose, and two of D-xylose are identified in a hydrolyzate of the methylated polysaccharide. Results from these structural investigations are reduced in value by a lack of knowledge of the number of polysaccharides in the preparation. Possibly, a heteropolysaccharide with L-arabinose and D-xylose units is present. If so, it would be similar to the polysaccharide isolated from wheat flour, which consists of a main chain of β -1 \rightarrow 4 linked D-xylopyranose units to which are attached single L-arabofuranose units through 1 \rightarrow 3 links (81). The origin of the methylated D-galactose derivatives is not known.

An acidic hemicellulose, which constitutes 75 per cent of the total hemicellulose of the fibre, can be isolated from jute chlorite holocellulose (83). Preliminary investigation suggests that there is one D-xylopyranose end unit and one monomethylglucuronic acid unit for each six nonterminal D-xylose units. The methylaldobiouronic acid has been isolated but not characterized. The affinity of the fibre for basic dyes is ascribed to the presence of this acidic constituent. There is said to be no free xylan in the jute fibre.

Cellulose.—One of the most interesting problems in this field at the present time concerns the possible chemical linkage which may exist between cellulose and the hemicelluloses (page 84). It is possible that those hemicelluloses which cannot easily be extracted from plant material by aqueous alkali are merely physically entrapped in the cellulose matrix though evidence is accumulating which suggests that a covalent union exists in some instances between the two types of coexisting plant polysaccharides. Such covalent attachment is suggested by the isolation from slash pine α -cellulose of a disaccharide composed of D-glucose and D-mannose units (84). The disaccharide is obtained by acetolysis. Control experiments show that this compound is not produced by "reversion" under the acetolyzing conditions.

Arabinose and xylose are present in α -celluloses derived from hardwood, and from wheat, jute, barley, and oat straws. Mannose and xylose are present in softwood α -cellulose hydrolyzates. Although these sugars have been identified only by paper chromatography, their absence from a cotton α -cellulose hydrolyzate is indicative of the validity of the experimental technique (85). The treatment of jute α -cellulose with 85 per cent formic acid for 12 min. at 135°C., followed by chromatography of the oligosaccharides produced, leads to the isolation of a trisaccharide which further hydrolysis shows to be constituted of glucose, arabinose, and xylose (86). Here again the evidence is solely from paper chromatography and thus is not conclusive. It might, however, suggest a chemical union between cellulose and pentosans.

Cellulose structures based on the results of earlier methylation experiments (87) must now be revised. Soluble O-methylcelluloses of D.P. from 250 to 1000 D-glucose units yield tetra-O-methyl-D-glucose end groups in quantities to accord with their osmotically determined D.P. The very slight discrepancy between the D.P. as measured by osmotic pressure and by end group content may be attributable to the presence of branched molecules. The maximum permissible number of branches from these data is not greater than one per molecule and further evidence is required before their presence can be taken as substantiated. Oxygen must be very vigorously excluded during the Haworth methylation of cellulose. If proper precautions are taken, O-methylcelluloses can be obtained from Egyptian cotton with degrees of polymerization determined by end group content of the same order as that of the starting material. Thus it is considered that the original cellulose has an unbranched structure (88).

A polymer homologous series of oligosaccharide α -D-acetates can be obtained from cellulose by acetolysis and chromatographic resolution (89). The unsubstituted members of the series from cellotetraose upwards exhibit the x-ray diagram characteristic of cellulose hydrate (90).

In recent years considerable attention has been given to the possibility that extremely acid sensitive linkages, "weak bonds," may be present equally spaced along the molecular chains of natural celluloses. The evidence for the existence of these "weak bonds" is based mainly on chain length distribution studies of degraded celluloses (91, 92). It is possible, however, to interpret the results from distribution experiments without postulating "weak bonded" structures. In the past insufficient attention has been paid to the effect of the crystallinity of the cellulose upon the kinetics of heterogeneous hydrolysis. By regenerating cotton nondegradatively from dilute solutions in alkali, the crystallinity can be reduced to an extent where the kinetics of hydrolysis can be followed under virtually homogeneous conditions. If the alkali treatment is carried out in the complete absence of oxygen the absolute rate constant of the highly amorphous cellulose agrees with that for normal β -1 \rightarrow 4 glucosidic bonds. "Weak bonds" of the types previously proposed are absent from cotton cellulose.

If oxygen is not completely excluded during the alkali treatment, a number (1 per 670 glucose residues) of acid sensitive, randomly distributed "weak bonds" are introduced. Thus a small fraction of abnormal linkages are present in cotton cellulose which may be rendered acid sensitive by alkaline oxidation. In unmodified cellulose 90 per cent of these linkages are protected by inclusion in the crystalline regions, and therefore escape detection (93).

Plant gums and mucilages.—Sapote gum, a heteropolysaccharide composed of D-xylose, L-arabinose, and D-glucuronic acid, is an exudate of a fruit tree found in tropical Central America. Some insight into its structure is obtained by identification of the methylated monosaccharides which result from methanolysis of the fully methylated polysaccharide (94). Distillation of the methanolysis products leads to the isolation of the methyl glucosides

of 3-O-methyl-D-xylose (2.8 moles), 2,3,4-tri-O-methyl-D-xylose (1.0 mole), 2,3,4-tri-O-methyl-L-arabinose (2.1 moles), and 2,4-di-O-methyl-D-glucuronic acid (1.1 moles). Free L-arabinose is not present in the gum so the pyranose ring structure found in the L-arabinose units must represent a true configuration within the gum structure. Methyl ester groups of the methylated polysaccharide can be reduced with lithium aluminum hydride, thus allowing the glucuronic acid to be determined as 3,4-di-O-methyl-D-glucose. From these data it is possible to draw only limited conclusions regarding the structure of sapote gum. All the L-arabinose and part of the D-xylose are present as end groups. Other D-xylose units may exist as branch points in the molecule with side chains attached through positions 2 and 4; the D-glucuronic acid is linked into the molecule through the seldom described 1→2 linkage.

The impossibility of characterizing a complex polysaccharide on the basis of methylation studies alone is further shown by results of work on the constitution of a polysaccharide from Tamarind seed (*Tamarindus indica*, Linn.). Separation, identification, and determination of the methanolysis products of the methylated polysaccharide yield the following data: 2,3,4-tri-O-methyl-D-xylose, 1 part; 2,3,4,6-tetra-O-methyl-D-galactose, 1 part; 3,4-di-O-methyl-D-xylose, 1 part; 2,3,6-tri-O-methyl-D-glucose, 1 part; and 2,3-di-O-methyl-D-xylose, 2 parts (95). End groups and branch points are thus identified, but suitable differentiation between various possible structures for this polysaccharide and also for sapote gum necessitates the isolation of fragments of the macromolecule such as may be obtained by partial acid or enzymatic hydrolysis.

4-O-Methyl-D-glucuronic acid was previously identified in a hydrolyzate of mesquite gum (96). The aldobiouronic acid, of which this sugar derivative forms the acidic component, is now characterized as 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose (97). A preliminary investigation of gum myrrh (98) shows that its components are D-galactose, L-arabinose, and 4-O-methyl-D-glucuronic acid, which occur in the proportions 4:1:3. The gum of the silk oak tree (*Grevillea robusta*) consists of the calcium and magnesium salts of an acidic polysaccharide composed of D-glucuronic acid combined with D-galactose and L-arabinose (99). Mild acid hydrolysis liberates all the L-arabinose together with some D-galactose, leaving a degraded gum. More drastic hydrolysis yields a simple aldobiouronic acid of D-galactose and D-glucuronic acid. Khaya gum from *Khaya grandifolia* (mahogany) and gum gatti from *Anogeissus schimperi* have been subjected to graded hydrolysis and their component sugars examined by paper chromatography (100). Hydrolysis of the former gum yields galactose and a degraded polysaccharide composed of galactose, rhamnose, and galacturonic acid. The latter gum, on hydrolysis, gives arabinose, galactose, and a degraded polysaccharide containing arabinose, galactose, and glucuronic acid.

Fructans.—It is suggested that fructans might be synthesized in the storage organs of some Compositae by the enzymic transfer to D-fructose residues to sucrose (transfructosidation). An enzyme is present in artichoke

tubers which will transfer D-fructose units from inulin to sucrose, yielding a trisaccharide and higher oligosaccharides (101, 102, 103). This enzyme system has not been as thoroughly investigated as that concerned in the synthesis of starch, and no evidence is available to show how large the D-fructose acceptor (the primer), or how small the D-fructose donor must be. Sucrose does not donate D-fructose to sucrose to any appreciable extent. This field of enzymic synthesis and degradation of fructans would seem to hold a great deal of promise for further investigation. Classical structural studies on inulins and levans (104 to 109) support the concept of enzymatic synthesis. Those fructans of higher plants which have been investigated consist of a chain of D-fructofuranose residues terminated by a nonreducing end group of D-glucose linked as in sucrose. As a result these polysaccharides show no reducing properties.

A strain of *Bacillus polymyxa* produces two polysaccharides when grown on a yeast extract-salts-sucrose medium. One of these is a levan which seems to be devoid of D-glucose. The polysaccharide is constituted of a chain of D-fructofuranose residues linked 2→6 with a limited number of branches involving 2→1 linkages (110).

Glycogen, amylopectin, and amylose.—The opinion is expressed (111) that there is no clear-cut distinction in nature between glycogen and amylopectin, since these two polysaccharides are synthesized by closely similar processes. Support for this contention is provided by enzymic investigations on a number of glycogens. It is concluded (112) that between the most divergent types lie a group of polysaccharides of intermediate properties, including certain bacterial and protozoal polysaccharides. Potentiometric measurements show that there is a sufficiently large difference between the iodine uptake of normal glycogens and amylopectins to distinguish between the two structure-types (113). Straight line relationships are observed between iodine concentration and iodine bound per unit weight of these glucans. The slopes of the lines obtained with glycogens differ markedly from those obtained with amylopectins.

Enzymic studies appear to occupy the center of attention in this field, and the majority of results leading to present concepts have been obtained by these methods. Meyer's bush-like structure (114) for amylopectins and glycogens has been well-supported and is now generally accepted. By the alternate action of muscle phosphorylase and amylo-1→6-glucosidase, amylopectin is degraded stepwise. On analysis of the phosphorylase limit dextrins with respect to percentage degradation and end group content experimental results approximate very closely to those required by the Meyer formulation (115). A similar conclusion is reached on the basis of the molar percentage of maltose plus maltotriose liberated from a β -amylase limit-dextrin by the debranching R-enzyme (116, 117). Maltose and maltotriose can arise only from exterior unbranched chains in the molecule, depending on whether they originally consisted of odd or even numbers of D-glucose residues.

Maltulose, 4-O- α -D-glucopyranosyl-D-fructose, is demonstrated in a sali-

vary α -amylase hydrolyzate of rabbit-liver glycogen (118). The hydrolysis products are fractionated on a carbon column and the disaccharide isolated in a 4.5 per cent yield. No control experiments are reported and thus it must not immediately be concluded that D-fructose is present in glycogen, though it is now apparent that this is a possibility.

Linear α -1 \rightarrow 4-dextrins with a minimum chain length of about 40 units are necessary for rapid transglucosidation by potato Q-enzyme to give α -1 \rightarrow 6 linkages (119, 120). A slow branching action is observed when this enzyme acts upon substrates of shorter chain length. Synthetic structures having properties intermediate between those of amylose and amylopectin can be isolated after short periods of incubation of amylose with Q-enzyme, which indicates that the action of this enzyme on amylose is random. The Q-enzyme of *Polytomella coeca* has the same properties (121).

The action of a sample of crystalline β -amylase is shown to be different from that of amorphous soya bean β -amylase preparations in that the former converts potato amylose into maltose to the extent of only 70 per cent. A further enzyme, designated Z-enzyme, is present in the amorphous preparation (122). Z-enzyme supplements the β -amylase in effecting complete conversion of the amylose into maltose. It is a β -glucosidase, its action on amylose being simulated by the β -glucosidase of sweet almonds. This enzyme will hydrolyze β -D linkages of the 1 \rightarrow 2, 1 \rightarrow 3, 1 \rightarrow 4, and 1 \rightarrow 6 type. It appears to operate by endwise attack on terminal glucosidic links as glucose was the only low molecular weight product identified in a laminarin hydrolyzate. The action of Z-enzyme on amylose is independent of β -amylase. A sample of amylose pretreated with Z-enzyme is hydrolyzed to a greater extent by β -amylase than is an untreated sample. It is suggested that these facts indicate the presence of single D-glucose residues joined to the amylose main chain by β -D-linkages (123).

Amylose is degraded under conditions similar to those normally used for the dispersion of starch. Samples of potato amylose are sealed in tubes with water or alkali under an atmosphere of air, oxygen, or hydrogen and heated for various times at 100°C. Determination of the intrinsic viscosity of each sample after treatment shows that in the presence of air or oxygen and in aqueous solution amylose is degraded slowly. The extent of the degradation is decreased in an atmosphere of hydrogen. In alkaline solution at 100°C. degradation is rapid unless oxygen is excluded (124).

In view of the "reversion" which takes place during treatment of sugars with acid, and which has been mentioned earlier in this review, it is necessary to prove that the 1 \rightarrow 6 linkage is preformed in amylopectin. This is accomplished by showing that the quantity of isomaltose isolated from an amylopectin hydrolyzate is 200 times in excess of the amount of this disaccharide produced by "reversion" when D-glucose is treated with acid under identical conditions (125). Gentiobiose and isomaltose are produced in approximately equal quantities by "reversion," under these conditions (0.082N HCl at 97°C.). The amount of "reversion" increases with the concentration of D-glucose.

Examination of maple sapwood starch (126) shows it to contain approximately 17 per cent amylose. Methylation work indicates one nonreducing end unit for each 26 sugar units in the amylopectin fraction. Periodate oxidation suggests one end unit for each 22 units.

Streaming dichroism and streaming birefringence can be used to measure the lengths of the amylose-iodine complex and of free amylose respectively (127). Results on a series of five subfractionated samples show a positive but surprisingly small correlation between the length of the complex and the intrinsic viscosity of the parent amylose. The mean lengths of 13 subfractionated amylose samples agree, in general, with the lengths of the corresponding iodine complexes. It is concluded that in the solvents used, glycerol-ethylenediamine and aqueous potassium hydroxide, the configuration of amylose is most probably the helical one rather than a random coil.

A value of 36 million \pm 10 per cent is reported for the molecular weight of potato amylopectin by light-scattering measurements (128). Fractions precipitated from aqueous solution by addition of ethanol have molecular weights varying from 52 to 7 million, with a weight average of 36 million. The particle diameters vary from 4300 to 2200 Å. Results for amylopectin acetates, after correction for acetyl content, agree with those values obtained for the same samples before acetylation. There is no indication of molecular aggregation of the substituted or unsubstituted amylopectin in many solvents.

Values for the molecular weight of the same sample of amylopectin acetate are quoted, varying from 420 million \pm 100 million as determined by light-scattering (129) to 6 million determined osmotically (130), a method which is known to be inaccurate in this region of molecular size. It is considered that those results first mentioned were obtained under the more ideal experimental conditions and therefore have the most foundation in fact.

Polysaccharides of lower organisms.—A number of polysaccharides from microorganisms are now being examined. The specific polysaccharide of *Pneumococcus* Type II is a heteropolysaccharide of complex structure (131). It contains D-glucose linked in positions 1, 4, and 6 (2 parts); L-rhamnose linked in positions 1 and 3 (8 parts); and D-glucuronic acid linked in positions 1 and 4 (1 part) and also as an end group unit (1 part). An interesting glucan can be isolated from *Aspergillus niger*. The structure of this polysaccharide is unique in that it consists of an unbranched chain with half the glucosidic links being of the α -1 \rightarrow 4 type and the remainder having the α -1 \rightarrow 3 configuration (132).

Polysaccharides have been isolated from the fresh-water algae, *Nitella*, *Oscillatoria*, and *Nostoc* (133). No carbohydrate is extractable from dried, powdered *Nitella* harvested in November. The residual polysaccharide is cellulose. Extraction of dried *Oscillatoria* powder with hot dilute alkali affords a mixture of polysaccharide and protein, the main carbohydrate component of which is a glucan. Methylation and identification of the hydrolysis products, together with iodine-staining reactions and optical rotation show this polysaccharide to be of the amylopectin type. It probably constitutes

the main food-reserve carbohydrate of this species of algae. A mucilage is obtained on extraction of *Nostoc* with hot water. It contains at least five sugars in addition to uronic acids. The stability of this polysaccharide to hydrolysis is such that a precise statement of the relative proportions of the constituents is not yet possible. It contains about 30 per cent hexuronic acids, 10 per cent L-rhamnose, 25 per cent D-xylose, and the remaining 35 per cent consists largely of D-galactose with smaller amounts of D-glucose and an unidentified sugar. A glucan is present in the flesh of the fungus *Polyporus betulinus*. Methylation studies indicate one nonreducing end unit for each 19 sugar residues and suggest that a high proportion of the D-glucosidic linkages have the 1→3 configuration (134). Mono-O-methylaldohexoses are found only rarely in nature, where they occur usually as the 3-O-methyl derivative. Recent work indicates that mono-O-methylhexoses, which are not 3-O-methyl derivatives may be present in small quantities in some soil polysaccharides (135).

Galactans.—A galactan (pneumogalactan) isolated from beef lung is the first to be obtained from mammalian tissue. D-Galactose is the only sugar detected after hydrolysis (136). The polysaccharide is alkali-sensitive and contains one titratable acid function, probably carboxyl, per 35 to 40 anhydrohexose residues. This sensitivity to alkali renders etherification difficult, but methylation can be accomplished by the thallium technique followed by the use of Purdie's reagents. Hydrolysis of the methylated polysaccharide yields approximately equal quantities of 2,4-di-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-galactose and 2,3,4,6-tetra-O-methyl-D-galactose. These sugars are identified as their crystalline anilides, which are prepared as a mixture from the methylated galactan hydrolyzate and subsequently separated chromatographically on Magnesol. Periodate oxidation indicates an uptake of 4 moles of oxidant per anhydrotrisaccharide unit, with the formation of 2 moles of formic acid. This polysaccharide therefore may consist of a chain of D-galactopyranose residues linked 1→6 and to every alternate unit in the chain is attached, in the 3 position, a D-galactopyranose end group. This is thought to be the first identification of a 1→3 linkage in a polysaccharide of mammalian origin. It is interesting to note that no hydrolytic enzyme could be isolated from extracts of lung, liver, kidney, duodenum, spleen, pancreas, guar bean, or brewers yeast. Many other enzyme preparations are also without effect on this polysaccharide. An unidentified soil bacillus can use the galactan for growth, but this may be an adapted behavior. An extract of sprouted lucerne seed showed weak hydrolytic activity.

Mucopolysaccharides.—A new method has been developed for the isolation of hyaluronic acid from human tissues. It is said to be simpler than methods used hitherto and depends upon extraction of the tissues with buffered trichloroacetate followed by precipitation with ethanol (137). A method for the determination of hyaluronic acid is based upon precipitation of the polysaccharide with hemoglobin at pH 3.8, and conversion of the prosthetic group of the precipitated protein to ferriheme cyanide. The intensity of the

color of this derivative is determined photoelectrically and the value for hyaluronic acid obtained by reference to a standard curve (138).

Evidence for the conversion of D-glucose to D-glucosamine and D-glucuronic acid by Group A streptococci is obtained by examination of the hyaluronic acid produced by these organisms when grown in the presence of 6-C¹⁴-D-glucose. The radioactivities of the sixth carbon atoms of the D-glucosamine and D-glucuronic acid moieties of the polysaccharide are found to be equal to that of the radioactive D-glucose (139). A similar conclusion has been noted previously as a result of experiments with 1-C¹⁴-D-glucose (140).

A complete picture of the structure of the hyaluronic acid molecule is not available at present. Isolation is reported of a crystalline disaccharide obtained from this polysaccharide in 61 per cent yield by enzymic and acid hydrolysis. This disaccharide is characterized as 3-O-(β -D-glucopyranosyluronic acid)-D-glucosamine (141). Formulation of hyaluronic acid as a chain of alternating N-acetyl-D-glucosamine and D-glucuronic acid residues must be, therefore, essentially correct. On the basis of periodate oxidation studies, Meyer (143) had characterized the glucosamine \rightarrow glucuronic acid linkage as 1 \rightarrow 3 and the glucuronic acid \rightarrow glucosamine linkage as 1 \rightarrow 4. Jeanloz & Forchielli (142), on the other hand, propose a uniform β -1 \rightarrow 3 linkage throughout the molecule. A still different structure is put forward by Blix (144). These differences of opinion make clear the necessity for further structural work on hyaluronic acid, using methods other than those of periodate oxidation.

The hyaluronic acid of ox synovial fluid occurs as a complex containing 25 per cent protein. The ratio of D-glucosamine to D-glucuronic acid is said to be 1.25 to 1.35. The complex is very easily degraded and only the mildest methods of separation (ultrafiltration and probably electrophoresis) preserve its native properties. The dynamic properties of solutions of the complex are consistent with the view that it is essentially a flexible chain polymer (145). The use of infrared spectroscopy as a tool for the elucidation of molecular structure of hyaluronic acid and chondroitin sulfuric acid has been discussed in a preliminary communication (146).

Chondroitin sulfuric acid is a heteropolysaccharide present in combination with protein in hyaline cartilage. Its component units are D-glucuronic acid (147) and N-acetylgalactosamine. A sulfuric acid group is attached to a repeating disaccharide unit, composed of one uronic acid and one amino sugar residue. This disaccharide, chondrosine, is obtainable in high yield by partial acid hydrolysis as a result of the resistance of the chondrosamine-glucuronic linkage. The uronic acid unit is therefore the reducing portion. Isolation of this disaccharide in high yield suggests a linear-type polysaccharide. Physical investigations of chondroitin sulfuric acid support this view (148, 149).

Although the glycosidic linkage of chondrosine was previously thought (150) to be 1 \rightarrow 3, it is now shown (151) to be β -1 \rightarrow 4, and the structural assignment is therefore 4-O-(N-acetyl-2-amino-2-deoxy- β -D-galactopyranosyl)-D-

glucuronic acid. Thus there are three free positions in the chondrosamine moiety to be occupied by the sulfate ester group and the linkage with glucuronic acid. Meyer proposes a 1 \rightarrow 6-union between glucuronic acid and chondrosamine (152). The latter conclusion is based upon the periodate uptake of a desulfated and deacetylated chondroitin sulfate. A considerable amount of degradation accompanies these two processes. The chain length falls from 60 to 6, and there is a fall in carboxyl content of 7 to 8 per cent. It is possible that there are other changes in molecular structure as a result of such treatments. The possible influence of the end groups on the periodate uptake does not seem to have been taken into account. The position of the sulfate ester group is still unknown.

Chondroitin sulfuric acid can be converted into a blood anticoagulant having approximately 50 per cent of the power of heparin by sulfation with sulfur trioxide (153). Direct evidence that sulfamic acid groups are a major factor in the anticoagulant activity of sulfated aminopolysaccharides is provided by the sulfation of chitin to yield a synthetic anticoagulant (154, 155). The contribution of sulfamic acid groups to anticoagulant activity is shown to far outweigh that of sulfate ester groups. The sulfamate groups of heparin are also thought to be responsible for inhibition of ribonuclease by this polysaccharide (156). It is found that heparin preparations containing a high proportion of sulfamic acid groups may have a relatively low anticoagulant activity. The loss of activity is not associated with extensive release of amino groups, hence it is possible that the activity of heparin may be a result of a combination of several factors, for instance, molecular size and shape as well as the degree of N-sulfation. The size and shape of the molecules could be profoundly influenced by the presence of cross links, and types of intermolecular cross linkages have been proposed. It is evident that degree of N-sulfation is not the single factor controlling activity but further investigation is necessary in order to obtain a clear picture of the relationship between chemical constitution and biological activity (157). In this connection, it is of interest to note that dextran sulfate, containing between 1 and 2 sulfate groups per D-glucose unit, has pronounced anticoagulant activity (158, 159).

The structure of heparin has been reviewed earlier (160). The presence of α -D-glucosamidic linkages, suggested previously on the basis of optical rotation data (161), is substantiated by studies on the rates of deamination of desulfated heparin by nitrous acid. The rates of deamination of the anomeric forms of methyl D-glucosaminide differ significantly. The rate observed for heparin is closely similar to that of methyl α -D-glucosaminide (162).

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NUCLEIC ACIDS

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Last year's review in this series emphasized the metabolic aspects of nucleic acids research. The flow of literature continues to affirm the great investigational enthusiasm evidenced for the many aspects of the field under study. This year's survey will undertake to cover certain chemical aspects for the period 1952 to November, 1953. References outside the period under review will be included in the bibliography only when required to maintain continuity in the text. A flood of evidence has shown the tetranucleotide theory and all of its correlations to be untenable. Therefore in the opinion of the reviewer all terms such as highly polymerized, depolymerized, pure and highly purified when applied in a descriptive sense to nucleic acids are highly misleading. Their use should be discontinued until such times as evidence is brought forth to justify the usage. The indications are that there are many nucleic acids. Certain data are highly suggestive that at least some of the nucleic acids are macromolecular, however there are other data which suggest that nucleic acids may be encountered with a molecular size as diverse as is known for proteins. No one sample of nucleic acid has unequivocally been shown to contain a single entity. At present writing it is seemingly misleading to assume as is done in many publications that the sample under investigation is other than a mixture of possibly many nucleic acids and their products of degradation.

PREPARATION AND PROPERTIES OF NUCLEIC ACIDS

Preparation of ribonucleic acids.—Recent methods for the preparation of ribonucleic acids are characterized by the complete abandonment of earlier drastic procedures in which the use of extremes in pH and prolonged treatment with heat are thought to have brought about chemical degradation. The characteristics of present procedures are, (a) extraction of nucleoproteins by the use of dilute salt solutions, (b) precipitation of the nucleoproteins at pH values no lower than 4.2, and (c) removal of the proteins by either strong salt solutions or surface active agents such as dodecyl sulfate or chloroform-octyl alcohol. In discarding drastic methods and accepting milder methods the way has been opened for enzymatic degradation and the relatively unstudied effects of concentrated salt solutions and surface active agents.

The fact that it was impossible to remove ribonuclease from a preparation of ribonucleic acids from pancreas by mild procedures and that degradation of the ribonucleic acids occurred during the procedures was first emphasized by Bacher & Allen (1). A similar association of ribonuclease activity with the nucleoproteins of the tobacco plant has been described by Pirie (2). A detailed study of the breakdown of ribonucleic acids in tobacco leaf extracts

and its relation to possible isolation of the nucleic acids is given by Parker (3). One of the mildest methods of recent introduction is that of Kerr & Seraidarian (4). The essential steps of the procedure are (a) extraction with 0.14*M* solution of sodium chloride, (b) precipitation of the nucleoprotein at pH 4.2, and (c) dissociation of the nucleoprotein by the use of a 3*M* solution of sodium chloride. The nucleic acid is then precipitated from alcoholic solution at neutrality. The product so obtained by its comparative behavior to dialysis shows ribonuclease contamination. Part of this contamination may be attributable to the formation and precipitation of a ribonuclease-nucleic acid proteinate such as is described by Kleczkowski (5). In order to minimize the possibility of enzymic degradation, Volkin & Carter (6) have incorporated the use of guanidine hydrochloride into a procedure, where it was thought that the denaturant action would be of aid both in the removal of protein and in the inhibition of ribonuclease activity. However, Simmons & Allen (7) have found that guanidine hydrochloride exerts little effect upon the crystalline ribonuclease from pancreas. A modification of the Volkin & Carter procedure has been published by Grinnan & Mosher (8). The modification introduces a short heat treatment after the guanidine hydrochloride denaturing step to aid in the removal of proteins. Thus it can be inferred that the chance of enzymic degradation is the same in both methods.

The most recent isolation technique has been proposed by Kay & Dounce (9). The essentially new feature is the gradual dissociation of the nucleic acids from the ribonucleoproteins by several treatments with sodium dodecyl sulfate. It is stated that sodium dodecyl sulfate "appears to block the enzymes which may partially degrade ribonucleic acid". The total time for the sodium dodecyl sulfate dissociation is 5 hr. Crestfield & Allen (10) have treated crystalline ribonuclease with sodium dodecyl sulfate under the conditions of Kay & Dounce after which time 40 per cent of the original ribonuclease activity remained.

The earlier literature offers no clue whereby complete inhibition of ribonuclease activity may be secured by mild methods nor does the present period offer much hope. Roth (11) and Zöllner & Fellig (12) have studied the inhibition of ribonuclease by heparin. The first study included the ribonuclease from pancreas, rat liver, and kidney while the latter concerned only crystalline ribonuclease from pancreas. The inhibition is too incomplete for use in the preparation of ribonucleic acids.

In view of the fact that most of the data for ribonuclease are available for that of the pancreas and since the little data we do have for the nucleases of other tissues indicate seemingly great differences, it may well be that the ribonucleic acids from certain tissues are much more easily prepared in an undegraded state than those of other tissues [see Maver & Greco (13, 14); Siebert, Lang & Corbet (15); Brown, Jacobs & Laskowski (16); Hilmoë & Heppel (17)].

Yields in the foregoing methods range from 20 to 50 per cent of the ribonucleic acids that can be assumed to be present on the basis of quantitative

analyses. There are no studies to give a clue as to the nature and reason for loss of such a major proportion. However, that the treatment of yeast both prior to and during isolation is of paramount importance is shown by Bourdet & Mandel (18). Autolysis if permitted to proceed for 10 minutes at 50°C. destroys 93 per cent of the nucleic acids originally present.

Analytical properties of the ribonucleic acids.—Researches of the period earlier than this review that can be classified in this category have been conducted mainly for the purpose of collecting data on the ribonucleic acids from as great a number of sources as possible in the hope of establishing some sort of identity each with the other. Except for a few laboratories insufficient attention has been given to the problem that is posed by suitability of isolation procedures and all of its possible relationships to the final product whose elementary composition was sought. As valuable as these researches have been it now appears that the problem of inhomogeneity and all of its implications must be given serious consideration.

In a series of papers Khouvine & Robichon-Szulmajester (19, 20, 21) have prepared from a baker's yeast three nucleoproteins termed *a*, *b*, and *c*. Each one was obtained by fractional precipitation at pH 4.96, 4.32, and 2.35, respectively. Three ribonucleic acids were prepared from the three nucleoproteins after drying with acetone. Chromatographic estimation of the nucleotide composition of each of the nucleic acids yielded a different proportion of nucleotides. However, if the yeast were dried with acetone prior to the direct extraction of the nucleic acids, a different nucleic acid, termed *d*, on the basis of its nucleotide composition could be found. Further, if the same yeast were dried with alcohol prior to the direct extraction of the nucleic acids then substances which resembled nucleic acid *b* in composition were found. The most probable interpretation of these data is that the methods of preparation have resulted in the fractionation of a large number of differently constituted ribonucleic acids into mixtures of nucleic acids and certain degradation products all of which cover a wide range of compositional and structural gradations. An attempt at the isolation of the ribonucleic acids fraction from the ribonucleoproteins of an atypical epithelioma of the rat leads to what is seemingly a badly degraded mixture [Khouvine & Hirsch (22)].

Loring and co-workers (23) have applied a newly described spectrophotometric method for the analysis of the purine and pyrimidine components of ribonucleic acids (24) to several samples of nucleic acids from yeast. Their results when compared with the results from other methods as applied in other laboratories lead the authors to conclude that considerable variation in composition occurs and that this variation is dependent upon the procedures that are employed during preparation and purification. It is stressed that the variations as noted may be attributable to (a) the occurrence of slightly different ribonucleic acids in the particulate components of cells, (b) partial enzymatic degradation, and (c) partial chemical degradation. Thus each possibility increases the degree of inhomogeneity that may be encountered.

The particulate components of liver tissue of the rat have been the subject of an investigation by Crosbie, Smellie & Davidson (25). The ribonucleic acids from mitochondria, microsomes, and cell sap are shown to have the same elementary composition but differ in composition from the ribonucleic acids of the nucleus. The carbohydrate moiety in all four samples was identified chromatographically as ribose.

Knight (26) has isolated the nucleic acids from six accepted strains and two provisional strains of tobacco mosaic virus. The molar proportions of the purines and pyrimidines in the accepted strains appear to be identical, but those of the provisional strains are different. The carbohydrate component, on the basis of chromatographic evidence is shown to be ribose by MacDonald & Knight (27). Eleven strains of tobacco mosaic virus were included in the latter study.

Quantitative estimation of ribonucleic acids and components.—Improvements in analytical methods and new techniques continue to appear. Loring *et al.* (24) have described a spectrophotometric method for the analyses of the purine and pyrimidine components of ribonucleic acids. The procedure involves the separation of the purines as silver salts after acidic hydrolysis. The pyrimidine nucleotides are converted to the corresponding nucleosides. It is claimed that, provided a small correction is made for the deamination of cytidylic acid, each nitrogenous constituent may be recovered with an accuracy of 99 ± 2 per cent. The method was developed in order to overcome certain lacks in precision in various of the paper chromatographic techniques as well as in other spectrophotometric procedures in which amongst other things the appreciably different molar extinction coefficients of the isomeric cytidylic acids offers difficulties.

The quantitative separation of a mixture of ribonucleotides by ionophoresis on paper has been achieved by Davidson & Smellie (28). Recoveries on the order of 93.5 to 102 per cent are claimed. The technique can be applied to the analysis of a ribonucleic acid after alkaline hydrolysis or to the ribonucleic acid fraction which is obtained from a tissue by a modification of the method of Schmidt & Thannhauser (29). Crosbie, Smellie & Davidson (25) have compared the foregoing procedure with two others which accomplish hydrolysis by the use of perchloric acid [Marshak & Vogel (30); Wyatt (31)] or hydrochloric acid [Smith & Markham (32)]. The procedure of Davidson & Smellie is concluded to be the most satisfactory. Details for the separation of purine and pyrimidine bases, the corresponding nucleosides and nucleotides as based on their ionophoretic behavior on filter paper strips are given by Werkheiser & Winzler (33).

An entirely automatic apparatus for separation by column chromatography and simultaneously recording the ultraviolet absorption of the components of ribonucleic acids has been described by Deutsch, Zuckerman & Dunn (34). Recoveries in the individual nucleotides of 102 per cent of the nitrogen present in the nucleic acid samples are reported (35).

Data on the chromatographic separation on filter paper of the components

of ribonucleic acids has been collected by Boulanger & Montreuil (36). Certain new single-phase solvent systems for the movement and separation of purines and pyrimidines as well as the corresponding ribonucleosides and ribonucleotides have been studied by Carpenter (37). A method whereby 1.5 to 5 μ g. of hydrolyzed nucleic acid may be analyzed for its components is offered by Edstrom (38). The determination of ribonucleic acids in molds and bacteria that are low in desoxyribonucleic acid has been accomplished by Dirks (39) by the use of 1N HCl for 10 min. at 60°C.

Preparation of desoxyribonucleic acids.—Largely as a result of their greater stability to alkali and to the fact that their nucleoproteins in many cases are easily obtainable the desoxypentose nucleic acids have seemingly not presented the same problems as the pentose nucleic acids. However it seems certain that many of the papers appearing during the present period are establishing a trend in thinking which will result in a complete revision of current ideas. During the last several years only two preparative procedures have been presented as representing distinct improvements over earlier methods. Marko & Butler (40) have been the first to apply the use of sodium dodecyl sulfate to the dissociation of desoxyribonucleic acids from nucleoproteins. Kay, Simmons, & Dounce (41) have improved the method such that no step is removed from neutrality nor are temperatures used higher than that of the room. Yields are 80 to 90 per cent of the total that is present. It may be well to note here that it was originally thought that the vexing problem of nuclease action was easily solved since pancreatic desoxyribonuclease had been shown to be inhibited by citrate ion. However it now appears that the desoxyribonucleases (14) have different properties. Hence here too the nuclease problem may well have to be solved for each individual tissue. Hurst, Marko & Butler (42) mention that some samples of desoxyribonucleic acids contain appreciable quantities of phosphomonoesterase activity which could not be removed by the usual deproteinization techniques of sodium dodecyl sulfate or chloroform-octyl alcohol.

Up until the past year there has been little evidence to suggest that the desoxyribonucleic acid as isolated from a given source was inhomogeneous. Bendich, Russell, & Brown (43) have succeeded in separating the desoxyribonucleic acids from several tissues of the rat into at least two fractions. Each fraction differs in its solubility in 0.87 per cent solution of sodium chloride and is metabolically dissimilar in that there is unequal incorporation of C¹⁴-formate in the two desoxyribonucleic acids from the same tissues. The metabolic evidence is the first of its kind and should stimulate further research for other evidences of inhomogeneity.

A report by Sherratt & Thomas (44) may have significance in this regard. A part of the desoxyribonucleic acids of *Streptococcus faecalis* are differentiated on the basis of their solubility in 1N solution of sodium hydroxide at 37°C. The two fractions do not vary significantly in their basic analyses.

Evidence of inhomogeneity in previous fractions of desoxyribonucleic acid from calf thymus has been reported by Chargaff and co-workers (45).

The fractional dissociation of calf thymus nucleohistone by the use of salt solutions of increasing concentrations has been found to yield a series of nucleic acid fractions. Most of the fractions are characterized by diminishing concentrations of guanine and cytosine and increasing concentrations of adenine and thymine. The authors emphasize that the equimolarity of each pair of constituents and of total purines and pyrimidines is maintained. The methyl cytosine composition of the various fractions shows significant divergence and this fact is offered as additional proof that the fractionation of the desoxyribonucleic acids of calf thymus has been achieved.

While not interpreted by their respective authors in terms of possible inhomogeneity of desoxyribonucleic acids, the following papers are highly suggestive of means whereby the problem may be studied at the nucleoprotein level. Bernstein & Mazia (46, 47) have studied certain properties of an undissociated desoxyribonucleoprotein, obtained by aqueous extraction of sea urchin sperm. It is shown that the characteristics of this nucleoprotein are quite different from the properties that are exhibited by nucleoproteins which are obtained by extraction in strong salt solution. Fleming & Jordan (48) have separated nucleoprotein from calf thymus into a fast and slow component. The separation was effected by following electrophoretic behavior at pH 7.1 to 12.0 and ionic strength of 0.02 to 1.0. It is noted that there is a marked decrease in nucleic acid content of the slow component with increasing pH until at pH 11.6 to 12.0 the component is pure protein.

Analytical properties of desoxyribonucleic acids.—A few years ago the literature contained a scarcity of data on desoxyribonucleic acids other than that from thymus. Recent years have witnessed great activity in this phase of research which is continued into the period under review. As a result of limitations in space little more can be done than cite certain of the studies which illustrate the great diversity of sources under investigation. Laland, Overend & Webb (49) have analyzed and compared desoxyribonucleic acids from calf thymus, herring testes, beef spleen, mouse sarcoma, wheat germ, *Mycobacterium tuberculosis*, and *Mycobacterium phlei*. Wyatt (50) has reported analyses to cover 11 insect viruses. Two different viruses from one host are noted to have distinct desoxyribonucleic acids. Chargaff, Lipshitz & Green (51) give compositions for four genera of sea urchins. Chromatographic evidence indicates the carbohydrate moiety in each instance to be desoxyribose. Gandelman, Zamenhof & Chargaff (52) have given data for three different strains of *Escherichia coli*; while Zamenhof, Brawerman & Chargaff (53) have done likewise for *Serratia marcescens*, *Bacillus Schatz*, and *Hemophilus influenzae*, Type C. Representations to the list for certain rickettsiae have been added by Wyatt & Cohen (54); bacteriophages T2, T4, and T6 by Wyatt & Cohen (55); an atypical epithelioma of the rat by Khouvine & Gregoire (56); and *Plasmodium Berghei* by Whitfield (57). Extensive coverage of the desoxyribonucleic acids from certain mammalian sources such as ox liver and thymus, sheep liver and thymus, pig liver, thymus, spleen

and thyroid, and human liver and thymus is reported by Chargaff & Lipshitz (58).

Discussion in many of the papers that are concerned with this phase of research center about the possibility of a common pattern for the desoxyribonucleic acids. This is best expressed in the finding that in many of the samples of desoxyribonucleic acids the molar ratios of adenine to thymine and guanine to cytosine plus methyl cytosine (when present) are approximately equal to one. It would seem that the experiences which released nucleic acid researches from the limitations which were imposed by the tetranucleotide theory should dictate extreme caution in applying significance in such a direction to data that are obtained by approximate methods of analyses to possible mixtures of what as yet are relatively unknown substances and their degradation products.

That dissociation of certain desoxyribonucleic acids from their nucleoproteins may be accomplished by dehydration, i.e., changes in the water lattice, has been shown by Fraser & Williams (59). Electron micrographs which are obtained by spraying a solution of bacteriophage T6 on to the grid, followed by freeze-drying and subsequent exposure to moisture, show such treatment to have effected release of the desoxyribonucleic acid. The nucleic acid takes the form of fine fibrillar strands. Diameters are approximated to be $20 \pm 5 \text{ \AA}$ by measuring the width of the shadows. Siegel & Singer (60) have succeeded in isolating and characterizing a desoxyribonucleic acid fraction from bacteriophage T2 by the use of a neutral buffer.

In a study in which the primary aim was to obtain more evidence concerning the possible desoxyribonucleic acid nature of the transforming substance of *H. influenzae*, Zamenhof, Alexander & Leidy (61) have obtained information which may in turn be of value to other projected studies on the desoxyribonucleic acids. For example, when subjected to heat the temperature at which transforming activity starts to decrease corresponds rather closely to the temperature at which the viscosity of the desoxyribonucleic acid fraction starts to decrease. Similar correspondence is found when the sample is subjected to changes in pH. Transforming activity is found to be reduced or destroyed by subjection to (a) low ionic strength, (b) dehydration, (c) desoxyribonuclease action, (d) sodium nitrite at pH 5.3 (e) formaldehyde, and (f) $10^{-6}M$ ferrous ion. Several protein denaturing agents are found to be without effect.

It should be noted here that a study of the immunologically active, type specific substance of *H. influenzae*, type b, by Zamenhof *et al.* (62) has shown that this substance consists of a polyribophosphate chain similar to that which is believed to be present in ribonucleic acids. In this instance the position of the purines and pyrimidines is occupied by a second similar chain of polyribophosphates which are believed to be linked to the first by means of 1:1'-glycosidic linkages. This substance is slowly broken down into dialyzable fragments by ribonuclease action and it is pointed out may have been

overlooked by having been classified amongst the ribonucleic acid fractions of cells.

A desoxyribonucleic acid fraction from a transplantable mouse lymphoma has been characterized with regards to a variety of physico-chemical properties by Shack and co-workers (63 to 66). The studies included, (a) electro-metric titration of the acidic and basic groups, (b) determination of the influence of electrolytes and of acids and bases on the viscosity, (c) determination of the variation of ultraviolet absorption spectra with pH, with concentration and type of added salt, and (d) estimation of the binding of the nucleate with ions by means of dialysis equilibrium and conductometric procedures. The behavior of this type of nucleate is noted as being somewhat similar to that of the nucleate from calf thymus which is the only other nucleate that has thus far been studied in a similar manner.

Frick (67) has shown that a sharp increase in the ultraviolet absorption spectra of thymonucleoprotein and desoxyribonucleic acid therefrom occurs at pH 11.0 to 11.3. The increase is 1.33 times the absorption noted in neutral solution. The change is attributed to alterations in the closely packed piles of purines and pyrimidines.

Complexes of lanthanum and other rare earths with desoxyribonucleic acid have been studied by Stern & Steinberg (68). Spectrophotometric and tracer methods have been used to determine their elementary composition.

Quantitative estimation of desoxyribonucleic acids and components.—Two papers have appeared in which desoxyribonuclease and phosphodiesterase have been used to achieve substantially quantitative degradation of desoxyribonucleic acids to desoxyribonucleotides. Both groups, Sinsheimer & Koerner (69) and Hurst, Marko & Butler (42) have effected final separation of the mononucleotides by the use of Dowex 1 columns. The latter authors have applied the method to the analysis of the desoxyribonucleic acid fractions from calf thymus, calf liver, calf pancreas, calf spleen, bull testes, chicken blood, human spleen, and wheat germ. Chromatographic systems which permit the separation of the desoxyribonucleosides from each other and from the desoxyribonucleotides are given by Tamm *et al.* (70).

A study of the Hunter reaction (71) by Day & Mosher (72) has resulted in the development of an improved colorimetric procedure for the quantitative estimation of thymine in hydrolysates of desoxyribonucleic acids. A micromethod based on the measurement of the density of color that is produced in the reaction between desoxyribonucleic acids and indole when heated in the presence of hydrochloric acid has been published by Ceriotti (73). The method is claimed to be applicable in ranges of concentration from 2.5 to 15 $\mu\text{g. per ml.}$

Quantitative fractionation and estimation of nucleic acids in biological materials.—Much of the literature concerning nucleic acids has dealt with possible amounts of the nucleic acids as believed to be present in some one tissue or particulate structure. The attainment of such results necessitates

the use of methods which far too frequently have only been tested on isolated fractions of the nucleic acids or a few common tissues. An awareness of the hazards that attend such indiscriminate application is the responsibility of the investigator who would undertake such adaptation. Of interest to those who plan to apply the Schmidt & Thannhauser (29) technique, Mauritzen, Roy & Stedman (74) have produced evidence that application of the method to isolated cell nuclei from a number of sources does not give a satisfactory separation of the two types of nucleic acids.

Davidson & Smellie (75) in an extensive study on the incorporation of radioactive phosphorus into the ribonucleotide fraction of liver tissue have concluded that the ribonucleotides comprise only about 75 per cent of the total phosphorus fraction which is obtained by the Schmidt & Thannhauser procedure. A modification is suggested. Sherratt & Thomas (44) when applying the Schmidt & Thannhauser technique to the separation of the nucleic acids fractions of a strain of *S. faecalis* found a large proportion of the desoxyribonucleic acid fraction to remain undissolved in the *N* NaOH used in the procedure and to remain firmly bound to polysaccharide like material.

Finally, Drasher (76) has confirmed the foregoing findings on mammary tumor tissue and raises the question of the general validity of the claim of Steudel & Peiser (77) that ribonucleic acids are hydrolyzed quantitatively to acid soluble nucleotides by treatment with 0.75*N* NaOH at room temperature in 24 hr. whereas desoxyribonucleic acids resist this treatment.

The possibility of estimating the nucleic acid contents of nuclei with a reasonable degree of accuracy has been the subject of a number of investigations. Aside from the chemical fractionation procedures referred to in the foregoing paragraphs, three other techniques are in common usage, (a) ultraviolet microspectrophotometry, (b) the staining technique dependent upon the Feulgen reaction, and (c) staining by the use of methyl green.

Chemical fractionation is of course dependent upon nuclear counting procedures if results are to be referred to content in terms of single nuclei. Albert, Johnson & Wagshal (78) have found that the adherence of large numbers of nuclei to the glass pipettes which are used to transfer nuclear material to the counting chamber results in abnormally low values for the number of nuclei present in the suspensions and in an erroneously high estimation in the desoxyribonucleic acid content per nucleus. The use of a wire loop for transference is proposed in order to circumvent the difficulties.

Most recent views with regard to the use of ultraviolet microspectrophotometry have been presented by Leuchtenberger *et al.* (79). The conditions that are necessary to insure quantitative results in the use of the Feulgen reaction have been the subject of a series of papers by Sibatani & Fukuda (80) and Sibatani (81, 82).

Frazer & Davidson (83) have undertaken a comparative study of the ultraviolet, Feulgen, and methyl green techniques on kidney and liver nuclei of the adult rat. It is concluded that provided the nuclei, which contains con-

siderable ribonucleic acid, is pretreated with ribonuclease ultraviolet measurements then present a general picture corresponding to that found by measurement of Feulgen and methyl green-stained material. Leuchtenberger *et al.* (79) consider the ribonucleic acid content of nuclei to be negligible in quantity and hence without effect on the ultraviolet absorption.

COMPONENTS OF RIBONUCLEIC ACIDS

Isomeric nucleotides.—The most stimulating and noteworthy contribution to the field of nucleic acids research in recent times is the now well known discoveries of the isomeric purine ribonucleotides by Carter & Cohn and of the isomeric pyrimidine ribonucleotides independently by Loring and co-workers and by Cohn. The solution of the nature of the isomerism has been a focal point in many investigations. The perplexing problem of the acidic and alkaline catalysis of migration of phosphate groups has been apparently solved by Brown, Magrath & Todd (84) by the preparation of the cyclic 2',3'-phosphates of adenosine, cytidine, uridine, and guanosine. The substances were separated chromatographically and examined in apparently homogeneous condition although not isolated in solid state. The cyclic phosphates are shown to be stable between pH 4.0 and pH 9.0 in aqueous solution. Treatment with 0.1*N* HCl for 1 hr. or 0.5*N* NaOH for 12 hr., both at room temperatures, effected hydrolysis to a mixture of the *a* and *b* isomers. Thus there can be no doubt of the role of the anhydrides as intermediates in acidic or alkaline hydrolyses.

In a study of the action of ribonuclease on the 2',3'-phosphates, Brown, Dekker & Todd (85) have shown cytidine-2',3'-phosphate to be converted into cytidylic acid *b*, and uridine-2',3'-phosphate into uridylic acid *b*. No action of ribonuclease could be observed on adenosine-2',3'-phosphate or guanosine-2',3'-phosphate. In the same paper it is demonstrated that alkaline deamination of cytidylic acid *b* yields uridylic acid *b*, hence it can be concluded that the phosphoryl group of both substances occupies the same position in the ribofuranose moiety.

Further clarification of the problem and its relationship to the nature of ribonuclease action is found in the work of Brown & Todd (86). Uridine benzyl phosphates *a* and *b*, barium uridine benzyl phosphate *b*, and cytidine benzyl phosphates *a* and *b* were prepared and subjected to the action of ribonuclease. Ribonuclease was noted to effect hydrolysis of the *b* isomers only, yielding uridylic acid *b* and cytidylic acid *b*. The corresponding cyclic nucleoside 2', 3'-phosphates are intermediates in the hydrolysis. The authors conclude that chemical and enzymic hydrolyses are similar with the exception that the enzyme is more specific in that only esters of the *b* isomers of the pyrimidine nucleotides are attacked.

The three following papers lead to the conclusion that the isomeric adenylic acids *a* and *b* are the 2'- and 3'-phosphates, respectively. By the phosphorylation of 5' trityl adenosine and removal of protecting groups,

Brown & Todd (87) have prepared two adenylic acids that are identical to the adenylic acids *a* and *b* as derived from ribonucleic acids. The evidence requires their formulation as adenosine-2'-phosphate and adenosine-3'-phosphate, although not necessarily in that order. Interconversion is postulated as attributable to facile phosphoryl migration in acid solution in analogy with the acid-catalyzed phosphoryl migration that is observed in the glycerol phosphates. Reichard, Takenaka & Loring (88) have fractionated the cyclohexyl ammonium salts of the two isomers of adenylic acid. Enzymatic dephosphorylation of the two isomers resulted in the formation of the same crystalline adenosine. Finally, Khym *et al.*, (89) have devised a procedure whereby it is believed that each ribose phosphate as hydrolyzed from an adenylic acid isomer can be directly identified as the component of that isomer and have concluded that the *a* isomer must be the 2' isomer and the *b* isomer the 3' isomer.

In a series of papers Loring and co-workers (90, 91, 92) have been active in the isolation and characterization of the isomeric cytidylic acids. As a result cytidylic acid $[\alpha]_D^{+18}$ is tentatively assigned the 2'-phosphate structure. Other isolation and characterization studies on the isomeric cytidylic acids have been reported by Harris *et al.* (93).

It has been noted by Cavalieri (94), and by Fox, Cavalieri & Chang (95) that in the pH range of 12 to 14 shifts occur in the ultraviolet absorption spectra of several pyrimidine nucleosides. This shift is attributed mainly to ionization of the 2' hydroxyl group of the carbohydrate moiety. In such cases the isomer that is substituted at position 2' would not exhibit the shift. A comparison of the ultraviolet absorption spectra for the two isomeric cytidylic acids shows the *a* isomer to be without variation in the pH range indicated. Hence it is concluded that the *a* isomer is the 2' isomer.

Since as noted previously (85) deamination of cytidylic acid *b* in alkali, which rules out possible phosphoryl migration, yields uridylic acid *b* then presumable uridylic acid *a* is uridine-2'-phosphate and uridylic acid *b* is uridine-3'-phosphate. At present writing the isomers of guanylic acid can only be termed by analogy.

Markham & Smith (96) have described the separation of the cyclic nucleotides from ribonuclease digests and from carefully controlled hydrolysates of the ribonucleic acids of yeast and turnip-yellow mosaic virus. The methods of paper chromatography and paper strip electrophoresis were used. Various properties are described, and it is noted that only the pyrimidine cyclic nucleotides are hydrolyzed by ribonuclease and at a very slow rate.

Nucleosides and dinucleoside phosphates.—The catalytic effect of lanthanum hydroxide on the dephosphorylation of nucleotides was first applied by Bacher & Allen (97). On the basis of a study of this catalysis on the hydrolysis of ribonucleic acids and nucleotides at various temperatures and mild pH, Shimomura & Egami (98) have proposed a method for the isolation

of nucleosides in quantity. When following the course of dephosphorylation by periodate consumption it was noted that periodate consumption precedes the dephosphorylation. Dephosphorylation studies on the individual mononucleotides, cytidine desoxyriboside, cytidylic acid *a*, cytidylic acid *b*, and 5'-cytidylic acid show the first three mononucleotides to be more readily hydrolyzed under the conditions than is 5'-cytidylic acid. Thus, from these data, the extra periodate consumption that is noted is attributed to the fact that the 2'- or 3'-phosphate is split off preferentially in comparison to that at the 5' position.

Markham & Smith (99) describe the isolation of four dinucleoside phosphates. These substances were obtained chromatographically by the use of phosphomonoesterase action on guanylyl-uridylic acid, guanylyl-cytidylic acid, adenylyl-cytidylic acid, and adenylyl-uridylic acid.

Nucleotide diphosphates and polynucleotides.—Enzymatic digestion of ribonucleic acid fractions from yeast, calf liver, and thymus and fractionation on ion exchange columns has resulted in the isolation of mixtures of the 2', 5', and 3', 5'-diphosphonucleosides of cytidine and uridine by Volkin and co-workers (100, 101, 102). The manner in which these findings are considered to bear on certain structural aspects are discussed by the authors and will be mentioned in a later section, c.f., page 115.

Merrifield & Wooley (103) have used ion exchange chromatography to isolate a number of dinucleotides and one trinucleotide from an acid hydrolysate of yeast nucleic acid. The hydrolysate was obtained by the use of 6*N* HCl for 3 min. at 25°C. after which the whole was poured into ice water. The nucleotides are claimed to have been obtained in analytically pure state and were characterized as 5'-guanylyl-cytidylic acid *b*, 5'-adenylyl-cytidylic acids *a* and *b*, 5'-cytidylyl-adenylic acid *b*, 5'-cytidylyl-cytidylic acid *a*, 5'-cytidylyl-cytidylic acid *b*, uridylyl-cytidylic dinucleotide, and adenylyl-diguanylyl acid trinucleotide.

The monumental task of separating 15 of the smaller polynucleotides which result from the action of ribonuclease on the nucleic acids from yeast and turnip-yellow mosaic virus has been achieved by Markham & Smith (99). The methods which were used involved preliminary chromatographic separation followed by paper electrophoresis. It is not clear whether all 15 were obtained from each ribonucleic acid fraction or if the 15 represent all possible small nucleotides as obtainable from the two types of nucleic acids. Upon characterization by the use of electrophoretic mobility, phosphomonoesterase action, and acidic or basic hydrolyses the nucleotides are concluded to be adenylyl-cytidylic anhydride, adenylyl-uridylic anhydride, uridylyl-uridylic anhydride, adenylyl-cytidylic acid, adenylyl-uridylic acid, adenylyl-guanylic acid, guanylyl-cytidylic anhydride, guanylyl-uridylic anhydride, guanylyl-cytidylic acid, guanylyl-uridylic acid, diuridylyl-uridylic anhydride, adenylyl-cytidylyl cytidylic anhydride, diadenylyl-cytidylic acid, diadenylyl-uridylic acid, and adenylyl-guanylyl-uridylic

acid. Attention is directed to the fact that all of the polynucleotides which remain after the completion of ribonuclease action with the exception of adenylyl-guanylic acid contain one and only one pyrimidine moiety per molecule. The pyrimidine nucleotide is in the terminal position in the molecule which it is pointed out is consistent with present information concerning the nature of ribonuclease action. The adenylyl-guanylic acid is regarded as an artifact of chemical degradation.

Smith & Allen (104) have separated six polynucleotides from an alkaline hydrolysate of ribonucleic acid from yeast under conditions which heretofore have been considered to bring about complete hydrolysis of internucleotide bonds. The polynucleotides have been separated by the use of paper chromatography and characterized only on the basis of their molar ratios of mononucleotide components.

COMPONENTS OF DESOXYRIBONUCLEIC ACIDS

A new pyrimidine base has been isolated from the desoxyribonucleic acids of T-even bacteriophages and identified as 5-hydroxymethylcytosine by Wyatt & Cohen (105). Comparisons with synthetic 5-hydroxymethylcytosine show the same ultraviolet absorption spectra and chromatographic behavior. An examination of the desoxyribonucleic acids from thymus and *E. coli* fails to reveal the new pyrimidine in these nucleic acids.

Desoxyribonucleosides.—The desoxyribonucleosides that are obtainable from a desoxyribonucleic acid fraction of thymus by intestinal nucleotidase action have been prepared and studied by Manson & Lampen (106). In this case the desoxyribonucleosides are those which contain guanine, hypoxanthine, thymine, and cytosine. Ultraviolet absorption curves are given. Metaperiodate studies indicate the four nucleosides to be furanosides. An examination of the cysteine-sulfuric acid reaction (107) shows that guanine desoxyriboside and hypoxanthine desoxyriboside react essentially quantitatively. Thymidine reacts partially, whereas cytosine desoxyriboside does not react under the specified conditions. The authors therefore suggest that only qualitative significance should be attached to data that are obtained with this method unless the composition of the desoxyribonucleic acid under investigation is known.

Anderson, Dekker & Todd (108) have presented a method which is suitable for the large scale preparation of desoxyribonucleosides. The method involves extraction with alcohol and ion exchange separation of an intestinal phosphatase hydrolysate of commercial herring sperm desoxyribonucleic acid. Silver salts are added to inhibit the adenine desoxyriboside deaminase. The desoxyribosides of guanine, adenine, cytosine, thymine, and uracil were isolated. The uracil desoxyriboside is believed to be present in the original desoxyribonucleic acid as a result of commercial preparative treatments. A new purine desoxyriboside, xanthine desoxyriboside, has been isolated and characterized as the cyclohexyl amine salt by Friedkin (109). It is formed

enzymatically from xanthine and desoxyribose-1-phosphate by the action of the xanthosine phosphorylase which is present in rat liver. Drell (110) has used a chromatopile to separate and isolate thymidine from an enzymatic digest of desoxyribonucleic acid from fish sperm.

Desoxyribonucleotides.—Thymidine-3'-phosphate and thymidine-5'-phosphate have been synthesized by Michelson & Todd (111). Upon comparison, thymidine-5'-phosphate was found to be identical to the thymidylic acid which is obtainable by the enzymatic hydrolysis of desoxyribonucleic acids. This confirms the views expressed by Carter (112) regarding the location of the phosphoryl group in natural thymidylic acid. Mild acidic hydrolysis of the desoxyribonucleic acids from herring sperm has been shown to yield thymidine-3', 5'-diphosphate and desoxycytidine-3', 5'-diphosphate [Dekker, Michelson & Todd (113)]. This confirms the original claim by Levene & Jacobs (114). Identity is established by comparison with synthetic products which were prepared by the phosphorylation of thymidine and desoxycytidine. Also confirming an earlier report by Cohn (115) desoxy-5-methylcytidine nucleotide is believed to be detected in the hydrolysate.

Several dinucleotides have been isolated from amongst the products of the action of pancreatic desoxyribonuclease upon desoxyribonucleic acids from calf thymus by Sinsheimer & Koerner (116). Those which were identified by ion exchange separation include a di-desoxycytidylic acid and a dinucleotide which contains desoxycytidylic acid and desoxyadenylic acid in equimolar proportions. Smith & Markham (117) have treated desoxyribonucleic acids from herring sperm with desoxyribonuclease and prostatic monophosphatase and claim to have identified dinucleoside monophosphate diesters which contain the combinations of adenine and cytosine, adenine and thymine, guanine and thymine, cytosine and thymine, thymine and thymine and finally, cytosine and cytosine.

INTERNUCLEOTIDE LINKAGES

Ribonucleic acids.—The several years preceding the period of this review have seen the documentation of two important facts without which an understanding of internucleotide linkages would be greatly hampered. The fact that the isomeric ribonucleotides appear as the result of the controlled alkaline hydrolysis of ribonucleic acids and that 5'-nucleotides are produced from the same ribonucleic acids as a result of diesterase action are well known contributions by Cohn and co-workers. It remained for Brown & Todd (118) to take account of these facts and apply the earlier known work on intramolecular phosphate migration to the nucleic acids. As a result of these considerations, Brown & Todd have formulated several types of nucleotide sequences in which the nucleoside residues are represented briefly as $C_2'-C_3'-C_5'$ (see Fig. 1).

The prerequisite for alkali lability in the ribonucleic acids is the presence of an hydroxyl group such that the cyclic anhydrides (84) can be formed

as intermediates in the hydrolysis. This is represented schematically in formulas A \rightarrow A' in which the proposed linkages in a phosphodiester sequence are represented as 3'5'. In addition, other structures which contain a C₂-C_{5'} or C₂'-C_{3'} repeating unit (C) or C₂'-C_{2'} and C₃'-C_{3'} units (D) would all show lability to alkali on the basis of intramolecular phosphoryl migration. A C₅'-C_{5'} linkage is believed not to occur since it would be stable to alkali and give rise to the appearance of dinucleotides in ribonucleic acid hydrolysates.

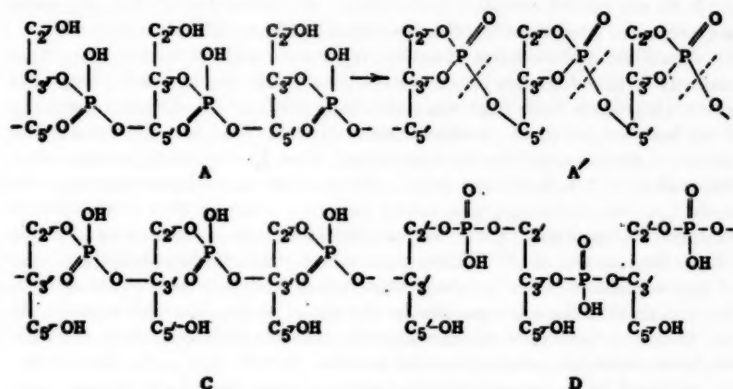


FIG. 1. Possible nucleotide sequences in ribonucleic acid.

Since formulas C and D take into account lability to alkali but not the fact that 5' nucleotides result from diesterase action, formula A which has the possibility of both 2'-5'- and 3'-5'-phosphoryl linkages is considered to be most probable for the main linkages.

In like manner the attachment of branches to the main phosphodiester sequence is thought to occur at 2' with the linkage through the phosphate group to 2' or 3' in the first nucleoside residue of any branch. Linkage at 2' in the main sequence to 5' in the first residue is considered as being too alkali stable since intramolecular phosphate migration would be impossible. The attachment of branches by means of phosphotriester linkages is thought to present no problem as the grouping would be alkali labile. Stability to alkali is believed to rule out the possible occurrence of 5'-5'-linkages.

In the absence of kinetic studies which would give strict definition to the terms lability and stability as applied to the internucleotide linkages, it is difficult to appraise the extent to which the linkages of the ribonucleic acids must be said to conform to the labile pattern. On the basis of the isolation work already mentioned a major part of the linkages that are present in the ribonucleic acids so studied must conform. However, Cavalieri (119) in a study on the kinetics of the alkaline hydrolysis of ribonucleic acids from

yeast concluded that on the basis of the velocity constants there appeared to be at least two types of linkages present, one much more labile than the other. This is supported by a later study along the same lines by Bacher & Kautzmann (120). These latter authors found the entire content of diesterified phosphate to be hydrolyzed according to a first-order rate law under some but not all conditions, hence it could not be concluded that all of the internucleotide linkages are of the same type. That all samples of ribonucleic acids do not exhibit complete hydrolysis of all internucleotide linkages under a given set of conditions has been recorded by Zittle (121).

As still further evidence of variability toward alkaline hydrolysis certain recently initiated studies by Hakim & Allen (122) may be cited. Samples of ribonucleic acids from pancreas and yeast, prepared by different methods, have been subjected to alkaline hydrolysis under identical conditions. The liberated mononucleotides have been estimated by chromatographic procedures after 1, 2, 4, 8, 15, and 24 hr. of hydrolysis. Early in the course of the hydrolysis the chromatograms take a definite pattern in that in addition to the positions occupied by the mononucleotides there are from seven to nine (depending on the time) definite areas which presumably contain mixtures of polynucleotides. The polynucleotide areas have not been characterized, but certain of them are separable by the use of electrophoresis on paper. As the hydrolysis proceeds the concentrations in the polynucleotide areas diminish while those of the nucleotides increase. At 15 hr. the extent of hydrolysis as based on mononucleotide formation ranges from 73 to 90 per cent. Hydrolysis at 24 hr. ranges from 95 to 98 per cent.

There are several reports in the literature that are difficult to reconcile with the statement that only isomeric mononucleotides are produced as a result of controlled alkaline hydrolysis. Allen (123) and about the same time Cavalieri, Kerr & Angelos (124) reported that the action of ribonuclease on ribonucleic acids from yeast yielded substances which gave a small but significant titre with periodate. Later data by Becker & Allen (125) in which lead tetraacetate was used in place of periodate confirmed the finding that certain of the smaller polynucleotides resulting from ribonuclease did indeed show a significant number of free glycol configurations. Further, if the same small polynucleotide fraction was hydrolyzed in 1N NaOH, a small but significant increase in the number of free glycol configurations occurred.

In order to test the possible occurrence of 5'-5' linkages in ribonucleic acids, Elmore & Todd (126) have synthesized adenosine-5'-uridine-5'-phosphate. This dinucleotide was found to be stable to treatment with 0.5N NaOH at 37°C. for 16 hr., conditions which it is stated "effect the complete hydrolysis of ribonucleic acids to mononucleotides." It is further stated that "The absence of the C_3-C_4' internucleotide linkage in ribonucleic acids is thus proved, since such linkages if present would remain unbroken and products other than simple nucleotides would result."

It is mentioned earlier in this review that ribonuclease hydrolyzes phos-

phodiesterases containing 3' linkages of uridine and cytidine to 3' nucleotides (86). The problem for certain of the purine nucleotide linkages has been undertaken by Heppel, Markham & Hilme (127). For this purpose the action of spleen nuclease on the nondialyzable fraction resulting from ribonuclease action has been studied. It is determined that at least 90 per cent of the purine residues were liberated as *b* nucleotides. Thus, presumably, the major proportion of the purine internucleotide linkages are of the 3'-5' type.

Whitfield & Markham (128) have outlined conditions which they believe will permit deductions regarding the nucleotide sequence in a polynucleotide chain. The conditions are (a) dephosphorylation by the use of prostatic monophosphatase, (b) oxidation of the resulting 2'-3' glycol by the use of periodate, and (c) hydrolysis of the dialdehyde at pH 10.0 for 6 hr. at 37°C. The success of the procedure depends upon the stability of internucleotide linkages to alkaline hydrolysis. In this instance the authors claim that they are stable.

The subject of possible branching of nucleotides in ribonucleic acids has received considerable mention. Anderson *et al.* (129) have proposed the use of methylation as a means of approaching the problem. From their results it appears that triply, doubly, and singly phosphorylated residues could be present in the ribonucleic acid fractions from yeast. Such conclusions involve the assumptions that (a) all available hydroxyl groups are methylated, (b) no degradation occurs as a result of methylation procedures, and (c) any incomplete recovery of carbohydrate that is incurred in working up the hydrolysate involves loss of each fraction to approximately the same extent. After careful consideration the authors believe the assumptions to have been justified. Schmidt *et al.* (130) suggest that data which has been obtained by the titration of ribonuclease digests supports a possibly debranching action by that enzyme.

Cohn & Volkin (102) regard their finding of cytidine and uridine 2', 5'- and 3', 5'-diphosphonucleosides together with 3' pyrimidine nucleotides to indicate that branching occurs in the ribonucleic acids from yeast, calf liver and thymus. The diphosphonucleosides are considered to have their origin in branch points which are composed of 2', 3', 5' triphosphopyrimidine ribosides. The pyrimidine nucleotides are thought to originate from branches of but one nucleotide in length involving triply esterified phosphate. This interpretation is given to the data from a comparative study of the products of digestion of the various ribonucleic acids by crude snake venom and a purified diesterase preparation (131) of the same venom from *Crotalus adamanteus*. As a result of the importance of these findings and the dependence of their interpretation upon strict diesterase action the reviewer would suggest that both the ribonucleic acids and the purified diesterase preparation be assayed for possible contamination by ribonuclease. This suggestion is motivated by several observations (a) the *b* pyrimidine nucleotide results, (b) Taborda

and co-workers (132) have shown ribonuclease to be present in a number of snake venoms although *C. adamanteus* was not among those studied, and (c) the assay of two different samples of *C. adamanteus* venom in the reviewer's laboratory showed considerable activity in a recently purchased sample (1952) and a lessened but demonstrable activity in the remains of a sample purchased in 1942.

It might be well to mention at this point another recent announcement by Brawerman & Chargaff (133, 134) which might conceivably add to the difficulties that are encountered in interpreting results that are obtained by the use of other than crystalline enzymes. These authors have found that phosphatase preparations from malt and prostate show the ability to phosphorylate both ribo- and desoxyribonucleosides to the corresponding 5'-phosphate. In the case of the preparation from malt 5'-nucleotides may also act as phosphate donors in the system.

On the basis of a study of the end groups, and the general nature of the nondialyzable fraction which results from ribonuclease action, Markham & Smith (135) concluded the ribonucleic acids from yeast and from turnip-yellow mosaic virus to be mixtures of many kinds of short chains. From estimates of the proportions of terminal groups the maximum chain length for the nucleic acid from yeast is given as 12 nucleotide residues while that for turnip-yellow mosaic virus is given as 53. Calculations of molecular weights from such data gives figures much lower than many of the figures given in the literature for so called undegraded ribonucleic acids. A discussion that is based on the relative dialyzability in the presence or absence of salts of certain fragments resulting from ribonuclease action taken together with data on the estimation of end groups for this fraction leads the authors to believe the fragments to be comprised of a mixture of polynucleotides three to five nucleotides in length.

The nondialyzable fraction that results from ribonuclease action on ribonucleic acids from yeast has been the subject of many studies. Only a few of these which bear directly on the foregoing study will be mentioned. Molecular weight estimations by sedimentation and viscosity on a nondialyzable fraction obtained by dialysis approaching zero ionic strength gave a figure of greater than 2000 (136). An analysis of the pyrimidine to purine ratio for the same fraction gave a value of 1 to 4.6 (137). Schramm, Albrecht & Munk (138) consider a nondialyzable fraction obtained in their laboratory to have a molecular weight from 3000 to 10,000. Estimations of the pyrimidine to purine ratio by Schramm & von Kerekjarto (139) gave approximately 1 to 10. Magasanik & Chargaff (140) have analyzed the fractions obtainable from many types of nucleic acids. Their ratios for two different samples of ribonucleic acids from yeast are 1 to 5.9 and 1 to 8.3. Seemingly there is great variability in both the original samples of nucleic acids and in the fractions that are obtainable after ribonuclease action.

Desoxyribonucleic acids.—The desoxyribonucleic acids appear to be

largely straight chain polynucleotides in which the main internucleotide linkage is 3'-5'. The stability of this linkage to alkali and lability to acid offers little hope that the characterization of nucleotide sequences through the isolation of small polynucleotides may be approached in this manner. By following certain suggestions in the older literature Tamm, Hodes & Chargaff (141) have succeeded in removing the purines from several preparations of desoxyribonucleic acids from calf thymus without impairment of the interpyrimidine ratios. The purine removal is accomplished by a controlled treatment with hydrochloric acid at pH 1.6 and 37°C. Some destruction of the original desoxyribonucleic acids occurs. The term apurinic acid is proposed for the substance to replace the older term thymic acid. Tamm & Chargaff (142) consider apurinic acid to have a molecular weight of about 15,000. This value was obtained from data on light scattering, sedimentation, and diffusion. A series of chemical studies on the properties of apurinic acid have moved Tamm *et al.*, (143) to propose a structure for the desoxyribonucleic acid from which apurinic acid has been derived. The structure is believed to be that of a chain in which nucleotide sequences that consist principally of pyrimidine nucleotides are followed by tracts in which purine nucleotides predominate.

GEOMETRIC STRUCTURES

As one follows the course of progress of research on the nucleic acids it is interesting to note how frequently a page is lifted from earlier research on the proteins. A consideration of the principal features of the x-ray patterns, atomic coordinates, and other available information has stimulated the workers of three different laboratories to submit structural formulae for the nucleic acids. Pauling & Corey (144) predict the molecule to be a three-chain helix. Such a giant molecule would be cylindrical with approximately circular cross section. There are three possibilities as to the composition of the part of the molecule that is closest to the axis. It may consist of purine-pyrimidine groups, the carbohydrate residues, or the phosphate groups. The reasoning presented by Pauling & Corey leads them to the choice of the phosphate groups. Both ribo- and desoxyribonucleic acids are considered to fit into the same structural considerations.

Watson & Crick (145) regard the Pauling & Corey configuration as unsatisfactory and propose a radically different structure for the salt of desoxyribonucleic acid. Their structure is formulated as two helical chains each coiled around the same axis. The two chains are related by a dyad perpendicular to the fibre axis. Each chain loosely resembles the Furberg (146) model no. 1 in as much as the bases are on the inside of the helix and the phosphates on the outside. The chains are held together by hydrogen bonds of the purine position 1 to pyrimidine position 1, and of purine position 6 to pyrimidine position 6. Thus, for example, if adenine forms one member of a pair on either chain, then the other member must be thymine; similarly for

guanine and cytosine. Until further clarification of this restriction is offered it would seem to the reviewer that the structure could not apply to those desoxyribonucleic acids in which the ratios of adenine to thymine or of guanine to cytosine is other than one. Of equal consideration is the possibility that those nucleic acids which do show a ratio of one may only do so because the analysis reflects average values of a mixture of nucleic acids of exceedingly variable ratios. Watson & Crick observe that it is probably impossible to build the structure if ribose were substituted for desoxyribose. Detailed studies of x-ray diagrams by Wilkins, Stokes & Wilson (147) and by Franklin and Gosling (148) show reasonable agreement between experimental data and the structure that has been proposed by Watson & Crick.

Jacobson (149) has recently commented that the Watson & Crick formulation bears a remarkable similarity to the structure of the tetrahedral water lattice which in turn makes it possible to explain the great tendency of desoxyribonucleic acid to interact with water and also to interpret several strange properties of its solutions. The dimensions of the two helical polynucleotide chains is believed to fit well into the water lattice in which the interstitial space has a helical shape. Rowen (150) on the basis of light-scattering studies on a sample of desoxyribonucleic acid prepared according to Gulland, Jordan & Threlfall (151) believes the molecular configuration to be that of a slightly flexible rod of $M. 4.5 \times 10^6$. The molecular length was found definitely to be dependent upon the ionic strength, decreasing from 6800 Å to 4200 Å with increasing ionic strength. It is this contraction with its relationship to the water lattice which Jacobson believes explains why the osmotic pressure, the viscosity, and the dielectric constant all decrease rapidly when ionic strength is increased. Two recent electron micrographs of dried solutions of desoxyribonucleic acids show the molecules to be arranged in an irregular hexagonal manner [Williams (152); Liquier-Milward (153)]. Jacobson believes this to be explained by a long range ordering effect by the water lattice.

Wilkins *et al.* (166) have studied the x-ray diffraction photographs for several desoxyribonucleic acids and describe the main features of the structure as follows (a) the structure is helical, (b) a major part of the helix has one sharply defined diameter, (c) there are two coaxial 18 Å diameter helices spaced a half pitch-length apart, (d) spaced centrally between the two 18 Å diameter helices is one helix of mean diameter about 10 Å, (e) there are 11 nucleotides per turn of one helix, and (f) the nucleotide shape resembles that of a rod inclined to the helix axis.

A recent investigation on the surface films of a sample of desoxyribonucleic acid prepared by James & Mazia (154) according to the method of Mirsky & Pollister (155) has given data which fits the circular crosssectional dimensional requirements of both types of helical structures. When spread upon the surface of a 2M solution of sodium chloride the films are found to be monomolecular and approximately 22 Å in thickness.

Measurements of the intensity of light scattering from solutions of three deoxyribonucleic acids have been reported by Doty & Bunce (156). The nucleic acids were isolated from thymus by the methods of Gulland, Jordan & Threlfall (151), Schwander & Signer (157), and a modified procedure. Molecular weights which range from 4.0×10^6 to 6.7×10^6 are deduced from the measurements. The maximum dimension of the sample prepared by Schwander & Signer is given as 6400 Å. In the light of these figures earlier estimates of molecular weights by other methods appear to be low by about a factor of five. Northrup, Nutter & Sinsheimer (158) have determined the refractive increment of a sample of deoxyribonucleic acid from thymus and believe the foregoing data to be too high by approximately 37 per cent.

DESOXYRIBONUCLEASE ACTION

Information concerning the action of ribonuclease on ribonucleic acids has contributed immeasurably to our knowledge of the internucleotide linkages and certain other structural features of the ribonucleic acids. Thus it can be expected that studies on the action of deoxyribonuclease on the various deoxyribonucleic acids will, though slower to accumulate, eventually aid in an understanding of the deoxyribonucleic acids.

Several studies on the kinetics of deoxyribonuclease action have appeared. Gregoire (159) has measured the acidic groups that are liberated in the course of the hydrolysis of deoxyribonucleic acids from thymus. A spectrophotometric technique was used for the purpose. Gregoire & Gregoire (160) conclude that the activation of deoxyribonuclease by magnesium ions varies with the concentration of the substrate and that in the region of optimum concentration the reaction is monomolecular. Cavalieri & Hatch (161) have also studied the kinetics of the reaction by measuring the liberation of acidic groups. The authors claim their data to indicate a pronounced inhibition of the reaction by the products of the reaction. Hence it is suggested that the first products of the reaction must be nucleic acid-like in nature rather than small entities.

Somewhat similar conclusions have been reached by Rabatin, Friedland & Frajola (162). In this instance deoxyribonuclease activity was followed by the use of an optical centrifuge. The data are concluded to indicate that hydrolysis involves three measurable changes, (a) a change in shape, (b) a change in size in which a small number of like particles are produced, and (c) further cleavage into smaller fragments.

Tamm, Shapiro & Chargaff (163) have subjected "intact" deoxyribonucleic acid and several types of its fragments including apurinic acid to the action of deoxyribonuclease. The substances termed fragments were obtained from deoxyribonucleic acid (a) by treatment for 1 hr., at pH 11.8 and 25°C., (b) by treatment for 1 hr., at pH 2.95 and 25°C., and (c) by treatment for 25 hr., in a glycine buffer at pH 2.86 and 37°C. All of the foregoing men-

tioned substances except apurinic acid were hydrolyzed by desoxyribonuclease in the presence of magnesium ions to about the same extent and rate. However, if even a portion of the purines or their complete removal was effected as is the case with apurinic acid a complete block to desoxyribonuclease action was established. From other data obtained in the study it was interpreted that magnesium ions bring about a nonenzymatic disintegration of certain of the degradation products of desoxyribonucleic acids.

Bernheimer (164) has continued the study of the partial inhibition of Group A streptococcal desoxyribonuclease. The inhibitor is thought to be a streptococcal ribonucleic acid [Bernheimer & Ruffier (165)]. Crude and purified preparations of ribonucleic acids from a variety of bacteria have been found to inhibit the ribonuclease partially. Ribonucleic acids from pancreas, liver, tobacco leaves, wheat germ, and yeast failed to show inhibition. Two preparations of ribonucleic acids from tobacco mosaic virus were effective as inhibitors only in relatively high concentrations.

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CARBOHYDRATE METABOLISM

SOME ASPECTS OF THE INTERMEDIARY METABOLISM OF CARBOHYDRATES AND LIPIDS^{1,2,3}

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It is now recognized that the traditional division of metabolism into carbohydrate, lipid, etc. is no longer entirely appropriate to modern biochemistry. Not only have the broad fields become enlarged beyond the capacity of a single reviewer, but they lose their identity at the intermediary stages with the formation of common metabolites. In consequence, the Editors of this Review have indicated the desirability of breaking away from this traditional classification in favor of reviews covering smaller, more specific areas of metabolism. In line with this intent, the present review will deal most specifically with reactions involving the formation and utilization of acetyl groups. However, since this volume will not contain other reviews on carbohydrate or lipid metabolism, the scope of the present review has been extended somewhat to include certain other areas. As usual, the reviewer has had the unpleasant task of omitting far more citations than were included. To keep within the space allotted, it was necessary to omit such contiguous topics as polysaccharides, photosynthesis, electron transport, phosphorylation, nucleotide transformations, and in general papers dealing with specific enzymes or with metabolism in the intact animal. Many of the papers falling within these categories will doubtless be covered in other portions of this volume.

THE FORMATION AND UTILIZATION OF ACETYL GROUPS

The acetyl group, representing as it does the focal point for numerous dissimilative and synthetic processes, is particularly appropriate as a subject for review at the present time. The past few years have witnessed astonishing progress in our understanding of how the major metabolic fuels, viz., glucose and fatty acids, yield this active intermediate; at the same time the mecha-

¹ This review covers approximately the period, October, 1952 to October, 1953.

² The following abbreviations are used: AMP, ADP, ATP for adenosine mono-, di-, and triphosphate; DPN⁺ and TPN⁺ for di- and triphosphopyridine nucleotides; DPNH and TPNH for di- and triphosphopyridine nucleotides (reduced form); CoA or CoASH for coenzyme A (the esterification of CoASH with various acyl groups is indicated by replacing H with the appropriate group, e.g., CH₃COSCoA for acetyl CoA); FAD for flavin-adenine-dinucleotide; TPP for thiamine pyrophosphate; LTPP for lipothiamide pyrophosphate; POF for pyruvate oxidation factor.

³ The reviewer acknowledges with thanks the kindness of many investigators who supplied manuscripts in advance of publication.

nisms by which it is utilized for combustion or for synthesis of various cell components have also been prominent subjects of investigation.

β -OXIDATION

The broad outlines of the stepwise degradation of fatty acids by units of two carbon atoms were formulated 50 years ago by Knoop; however, it is only since the discovery of the participation of coenzyme A in fatty acid oxidation that real progress has been made in our knowledge of the individual enzymatic steps of this process. Our present picture of the catabolism of fatty acids to the acetyl state may be seen in Figure 1.

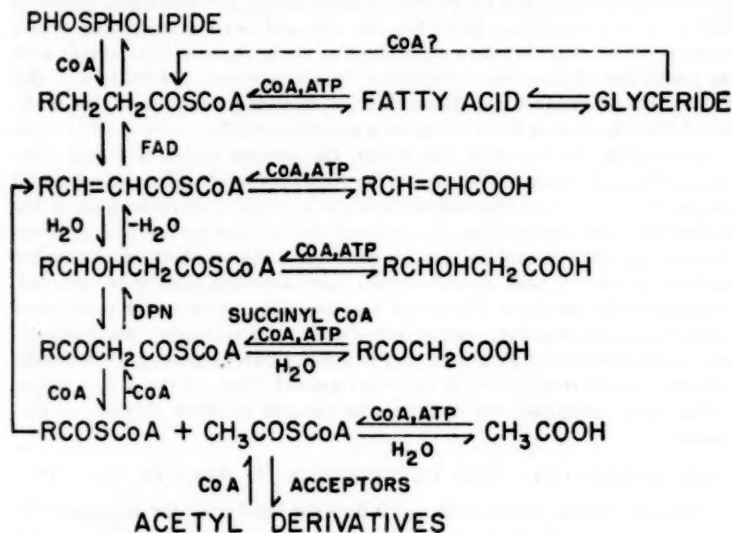


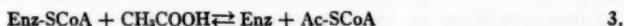
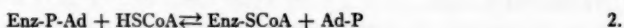
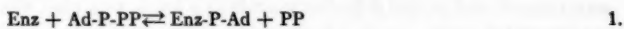
FIG. 1. CoA in the catabolism of fatty acids.

Much of our detailed knowledge of these processes followed shortly upon the discovery, by investigators at the Enzyme Institute of the University of Wisconsin, of means of "solubilizing" the enzymes of fatty acid oxidation. First reported in 1952 (1) and more recently described in greater detail by Drysdale & Lardy (2) was the successful preparation of a soluble enzyme system from acetone powders of rat liver mitochondria which, with the aid of artificial electron acceptors such as ferricyanide or 2,6-dichlorophenol indophenol, oxidized saturated fatty acids of chain length ranging from 4 to 18 carbons. The product was acetoacetate, and carboxyl- C^{14} -labeled acids yielded the keto acid labeled in both β and carboxyl carbons, indicating that the primary product was an acetyl group which secondarily condensed to

acetoacetate. A stoichiometric requirement for ATP³ was found, suggesting that after initial activation the successively shorter fatty acids do not require additional activation. In the presence of oxalacetate the oxidation of hexanoic acid yielded citrate but no acetoacetate; thus the condensing enzyme in this system competes successfully, in the presence of oxalacetate, for the acetyl groups formed by β -oxidation. That the first step in the process is the formation of a CoA³ ester of the fatty acid was suggested by formation of hydroxamic acid when hydroxylamine was added to the system, with or without an electron acceptor, and by demonstration of a CoA requirement when enzyme preparations were treated with Dowex-1, which removes CoA.

Acetone powders of liver or of isolated mitochondria therefrom have proved to be a rich source of material from which all of the individual enzymes of fatty acid catabolism have subsequently been isolated. Reviews of recent developments in this field have been published by Mahler (3) and by Lynen (4).

Activation of fatty acid ester formation with CoA.—Esterification of acetate and other fatty acids with CoASH has now been extensively studied. Apparently the original suggestion of Lipmann *et al.* (5) that the first stage is a reaction between ATP and CoA to yield a pyrophosphoryl-CoA ester was in error. More recent studies have not provided any evidence for the independent existence of such a compound. Using a purified acetate-activating enzyme from yeast, Jones *et al.* (6, 7) found that P³²-labeled pyrophosphate exchanged with ATP in the absence of CoA, and that this exchange was inhibited by CoA. This suggested that the reaction of enzyme with ATP leads to the reversible formation of pyrophosphate and an AMP²-Enzyme Complex, which can undergo exchange with CoA to yield free AMP- and Enzyme-SCoA. This in turn undergoes exchange of enzyme for acetate to yield acetyl-SCoA plus free enzyme. The reaction was formulated as follows:



Beinert *et al.* (8) studied the acetate-activating enzyme of rabbit or pig heart, which they also believe proceeds by way of a single enzyme, but apparently involves the intermediary formation of an ATP-CoA complex. Assay of the enzyme was carried out by coupling the formation of acetyl-CoA with hydroxylamine and measuring formation of acethydroxamic acid; by coupling with condensing enzyme and oxalacetate and measuring citrate formation; or less conveniently by spectrometric measurement of the absorption at 230 μ characteristic of thiol esters. The enzyme requires magnesium and potassium ions. In the presence of large amounts of CoA, acetyl CoA was prepared in quantity, the yield being limited only by the presence of coenzyme A deacylase, an enzyme which promotes the hydrolysis of acetyl

CoA to free acetate and CoA. The reversal of reaction 4 was demonstrated by coupling it with creatine phosphorylase and measuring formation of phosphocreatine from the ATP thus formed. The enzyme is specific for acetate (and propionate) and is inactive with ethyl acetate, thiolacetate, butyrate, acetylglycine, acetoacetate, and succinate. Von Korff & Glaman (9, 10) found this enzyme, which they termed acetokinase, to be stimulated by potassium, ammonium, or rubidium ions, and inhibited by sodium and lithium. A stimulating effect of potassium ions, which they observed on acetate oxidation in rabbit heart mitochondria, was attributed to their action on this enzyme.

According to Green & Mii (11), in addition to the acetate activating enzyme, there are several other enzymes which carry out the same reaction, but which differ in specificity toward acids of different chain length. A description of the properties of the butyrate-activating enzyme has been given by Wakil & Mahler (12). A similar enzyme activating the long chain fatty acids was reported by Kornberg & Pricer (13, 14). These investigators obtained a soluble enzyme preparation from guinea pig liver (but not from rat, mouse, chicken, or pigeon liver, or from other animal tissues, or crude extracts of yeast, *Escherichia coli* or *Clostridium kluyveri*) which carried out reaction 4 with a variety of saturated fatty acids ranging from C_4 to C_{22} , and oleic, linoleic, linolenic, and chaulmoogric acids. The enzyme was used, with stoichiometric amounts of CoA, to synthesize palmitoyl-SCoA.

From more recent results of Mahler *et al.* (15) it appears that the intermediate chain length fatty acids are activated by a single enzyme with a rather low substrate specificity. The enzyme prepared from hog or cattle heart, liver, or kidney acts on fatty acids ranging from 4 to 12 carbons and has greatest activity in the C_7 range. The relative activity toward fatty acids of different chain length did not change on purification. It activates unsaturated and α - and β -hydroxy-acids at a lower rate than the corresponding saturated acids, but has little or no action on β -keto or α -amino acids, or on succinic acid. It does, however, activate α -methylbutyric acid and such aromatic acids as benzoic, phenylacetic, and 2,4-dichlorophenoxyacetic. The products obtained from butyric and octanoic acids in the presence of hydroxylamine were shown by paper chromatography to be identical with authentic synthetic hydroxamic acids. The requirement for ATP could not be replaced by other nucleotides, and CoA could not be substituted by other sulfhydryl compounds such as glutathione, thioglycolic acid, cysteine, lactobacillus bulgaricus factor, or D,L-thioctic acid. The reactions are reversible with a K_{eq} in the direction of acyl CoA formation ranging between 1 and 8. Crude calculations yielded ΔF° values for hydrolysis of the CoA esters of the order of $-11,000$ cal./mole.

Recent data indicate that the physiological activation of fatty acids may not require the intermediate participation of the free fatty acid but may occur directly from an ester bond by transesterification with coenzyme A. Kornberg & Pricer (16) isolated an enzyme from liver which catalyzes the

formation of diacyl phosphatidic acid from acylSCoA and α -glycerophosphoric acid. Activity was highest with the saturated C_{16} and C_{18} acids, but reaction occurred with a wide variety of saturated and unsaturated acids ranging from C_{12} to C_{22} . Jedeikin & Weinhouse (17) observed, in extracts of acetone powders of pigeon liver, a coenzyme A-requiring system incorporating C^{14} -labeled palmitate into phosphatides. These investigators found that a respiration-dependent system which incorporates labeled palmitate into tissue phospholipids is present generally in organs of the rats. These findings suggest that a reversible system may operate in the transfer of fatty acids between CoA and intermediates in the buildup of the phosphatide structure. No evidence has yet been found for the attractive hypothesis that a similar mechanism may operate between CoA and glycerides.

Another possible means of activating fatty acids is represented by the CoA transphorase found in extracts of *C. kluyveri* by Stadtman (18). This enzyme catalyzes an exchange of CoA between acetate and a variety of fatty acids including C_1 to C_8 saturated acids, lactate, and vinylactate. The formation of formylSCoA by this reaction raises the possibility of the involvement of this ester in formylation reactions.

Desaturation of acyl CoA.—Evidence for the existence of an enzyme which reversibly desaturates fatty acids between carbons 2 and 3 (reaction 5) has been provided by Seubert & Lynen (19) and Green *et al.* (20). The former

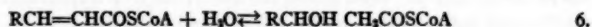


investigators have taken advantage of the fact that S-esters of N-acetylthioethanolamine can replace SCoA esters in enzymatic reactions. They purified a flavoprotein from sheep liver which oxidized leucosafrin in the presence of S-crotonyl-N-acetylthioethanolamine. The leuco dye could not be replaced by reduced DPN² or TPN². The ester could not be replaced by crotonic acid, but acted upon β -hydroxybutyrylSCoA in the presence of the hydase, described below, thus further implicating crotonylSCoA as the physiological substrate of this enzyme. A more detailed description of this enzyme and the reaction catalyzed thereby has been reported by Green *et al.* (20). This enzyme acts upon CoA esters of saturated fatty acids with chain lengths of 3 to 8 carbons, but exhibits greatest activity with butyryl CoA. It does not transfer electrons directly to oxygen, but will directly reduce cytochrome-*c*. For assay of the enzyme, spectrophotometric measurements of the reduction of 2,6-dichlorophenol indophenol, pyocyanine, and triphenyl-tetrazolium chloride, or cytochrome-*c* have been used. The equilibrium constant for the formation of butenoyl CoA is between 3 and 22, from which a value of E'_0 of 0.187 volts was calculated for the couple, butyryl CoA/butenoyl CoA. The products were not identified but were presumed to be the corresponding 2,3-unsaturated ester, on the basis of the activity of crotonyl CoA in the reverse reaction and in the next step, namely, the hydration to β -hydroxybutyryl CoA. It is not yet certain whether more than one desaturase exists, but it would seem, from the limited specificity of the enzyme

of Green *et al.* (20) that other analogous enzymes may be involved in the dehydrogenation of the higher acyl CoA esters.

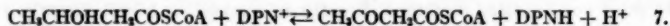
Mahler (21) reported that the prosthetic group of the butyryl CoA dehydrogenase is a cupri-flavoprotein, with a molar ratio of copper to flavin of approximately two in the purest preparations. He makes the interesting speculation that the copper mediates a one-electron transfer from reduced flavin to ferric iron of cytochrome.

Hydration of unsaturated acyl CoA ester.—Stern & del Campillo (22) assumed the occurrence of reaction 6, the hydration of the 2,3-unsaturated CoA ester, in ox liver and heart, on the basis of the observation that prepa-

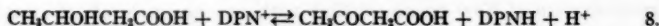


tions containing enzymes responsible for the subsequent reactions; viz. oxidation of β -hydroxybutyrate and cleavage of the resulting acetoacetate to acetyl CoA, acted upon crotonyl CoA in the presence of an acetyl acceptor (the citrate condensing enzyme was used) to yield citric acid. The properties of the enzyme catalyzing this reaction have been described by Wakil & Mahler (23). Assay of the reaction in the direction of hydration was carried out by coupling it with the DPN-linked β -hydroxyacyl CoA dehydrogenase (*vide infra*) and measuring the reduction of DPN⁺ spectrophotometrically. In addition to crotonyl CoA, the enzyme hydrated *trans*-hexenoyl CoA, and vinylacetyl CoA, but not *cis*-crotonyl CoA; evidently it is specific for *trans* esters. In the reverse direction the enzyme acted upon the β -hydroxy-CoA esters of butyric, hexanoic, octanoic, and lauric acids. The equilibrium of the reaction with C₄ acids is far toward the unsaturated ester, $K_{\text{eq}} = 2.8 \times 10^{-2}$. The fact that the β -hydroxyacyl CoA dehydrogenase and the hydase act only on the D-enantiomorph, coupled with the fact that only half of the D,L-ester is acted upon, was taken as evidence that the product of hydration is the D-hydroxy ester.

Dehydrogenation of β -hydroxy- to β -ketoacyl CoA.—An enzyme catalyzing the interconversion of β -hydroxy- and β -ketoacyl CoA esters was purified 300-fold from sheep liver by Lynen *et al.* (24). The reaction shown in equation 7 was followed in both directions by the change in absorption of the pyri-

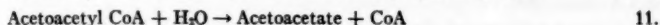
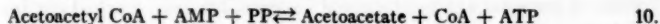


dine nucleotide. Study of this reaction was facilitated by the activity of the S-acetyl ester of N-acetylcysteamine, which made it unnecessary to prepare the CoA esters. With the initial addition of equivalent amounts of acetoacetyl ester and DPNH² at pH 7.35, the reduction to the hydroxy-ester was 95 per cent complete. Free acetoacetate or its ethyl ester were inactive. Lehninger & Greville (25) found that clear, dialyzed extracts of acetone-dried rat liver mitochondria contain two systems for oxidation of β -hydroxybutyrate. One is the well known DPN⁺-specific L- β -hydroxybutyric dehydrogenase catalyzing reaction 8; the other acts only on the D isomer, and requires

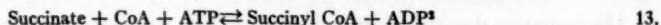
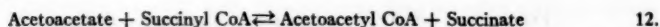


ATP, CoA, and Mg ions in addition to DPN⁺; the product is acetoacetyl CoA. The latter reaction occurs in two steps; the first is an ATP-promoted esterification of D-β-hydroxybutyrate with CoASH,² analogous to reaction 4 (established by trapping the ester with hydroxylamine); the second is the DPN-specific dehydrogenation to acetoacetyl CoA (equation 7) already described. This dehydrogenase has also been purified and studied by Wakil, *et al.* (26).

Acetoacetyl CoA formation and cleavage.—According to Stern *et al.* (27) and Green *et al.* (28) pig heart contains an acetoacetate formation and utilization system, distinct from that of liver, previously described. In liver these processes can be described in terms of the following equations.



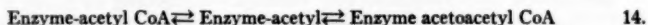
In the pig heart system, however, enzymes for reactions 10 and 11 are lacking, but acetoacetyl CoA can be formed by the reversible reaction shown in equation 12.



In the presence of succinyl CoA [which was prepared *in situ* either by the CoA, ATP reaction (equation 13) or by oxidation of α-ketoglutarate (*vide infra*)], acetoacetate was converted to acetyl CoA, whose formation could be followed by coupling with acceptors such as oxalacetate or hydroxylamine. Both groups agreed that acetoacetate was most active in this system, but other keto acids also were split to acetyl CoA and the CoA ester of the next lower fatty acid (identified by paper chromatography of the hydroxamates). Starting with acetyl CoA as such or generated *in situ*, the reaction could be reversed in the presence of succinate to drive reaction 12 to the left and to yield free acetoacetate. In contrast with the liver system, free acetoacetate is not formed spontaneously. It appears that the presence of the acetoacetyl CoA deacylase in liver may be the cause of the production of free acetoacetate and may be responsible for its generally low rate of metabolism in this organ. These findings thus provide a new dimension for speculation concerning relations between metabolic disturbances and ketosis. Stern *et al.* (27) pointed out that the formation of acetoacetate by fatty acid oxidation, by way of reversal of reactions 12 and 13 results in oxidative phosphorylation at the substrate level.

Beinert & Stansly (29) have offered an ingenious explanation for the often observed asymmetry in labeling of acetoacetate produced by liver slices from variously labeled fatty acids. They consider that the first step is a reversible combination of enzyme with acetyl CoA, followed by a reversible

condensation of Enzyme-acetyl CoA with another molecule of acetyl CoA. If the second reaction is faster than the first, the head moiety of acetoacetate will exchange with other acetyl CoA molecules more rapidly than the tail moiety; and if one makes the further reasonable assumption that the terminal C_2 unit splits off as an Enzyme-acetyl CoA complex it is not difficult to see why terminal acetyl groups preferentially form the tail moiety of acetoacetate. By analogy with other transferase reactions, it appears that in this transfer, an acyl-enzyme complex reversibly exchanges CoA for acetyl CoA as shown in equation 14. A somewhat similar explanation, involving the

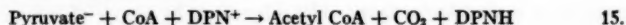


preferential acetylation activity of an Enzyme-acetyl complex has been offered by Lynen (4); and a means of predicting the distribution of C^{14} in acetoacetate from variously labeled fatty acids, based on the differing activities of terminal and nonterminal C_2 units, has been worked out by Brown & Chaikoff (30).

Using a mixture of the soluble, purified enzymes just described, together with malate, DPN^+ , malic dehydrogenase (to generate oxalacetate), and the citrate condensing enzyme, Beinert *et al.* (31) carried out the complete conversion of butyrate to citrate

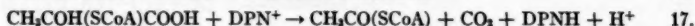
FORMATION OF ACETYL GROUPS FROM PYRUVIC ACID

The oxidative decarboxylation of pyruvic acid to yield acetyl CoA and CO_2 now emerges as an exceedingly complex process, involving the participation of at least five cofactors, viz., thiamine, α -lipoic acid, coenzyme A, DPN^+ , and Mg ions. It became well established several years ago that the oxidative decarboxylation of pyruvate in a variety of microorganisms and animal tissues occurs by a process which can be represented in the following over-all equation (equation 15).



The background of information on which this formulation is based may be found in previous volumes of the Annual Reviews (32, 33) and in reviews by Gunsalus (34, 35) and Schweet *et al.* (36). Briefly summarized, it consists of studies in which soluble enzyme preparations from a variety of bacteria (37 to 40) and several animal tissues (41 to 44) were found to oxidize pyruvic acid to acetyl CoA and CO_2 . In general, acetyl CoA was not determined as such in these preparations, but its formation was inferred by "trapping" acetyl groups as the hydroxamate, or by formation of acetyl phosphate, acetylsulfanilamide, or citric acid in the presence of the appropriate acyl transfer system.

The logical proposal was made at that time by Lynen & Reichert (45) that CoASH combined with the carbonyl group of pyruvate, and the oxidation of this complex (presumably by DPN^+) led directly to acetyl CoA, as shown in equations 16 and 17.



However, later work demonstrated that CoA functions neither in the initial activation and decarboxylation of pyruvate, nor in the generation of the acetyl group therefrom. In the meantime, considerable progress was made in our understanding of the functions of thiamine pyrophosphate and the so-called pyruvate oxidation factor⁴ in this process, and it now emerges that these substances are the cofactors directly concerned in decarboxylation and acyl generation.

Evidence that coenzyme A is not obligatorily involved in the oxidative decarboxylation of pyruvate by animal tissues was obtained by Schweet *et al.* (36, 46), who made the important observation that with ferricyanide as an electron acceptor, the only requirements for pyruvate oxidation in a soluble oxidase of pigeon breast muscle were Mg ions and cocarboxylase. Added DPN⁺ and CoA had no effect, nor could any evidence be obtained for the presence of either of these substances in the purified system. However, it did contain the pyruvic oxidase factor, the content of which increased as the enzyme system was purified. In the absence of CoA, the products of the reaction were acetate and CO₂; if CoA was present, however, acetyl CoA was formed, and when coupled with the corresponding acceptor system, acetyl-sulfanilamide was generated. In studies of pyruvate oxidation in *Proteus vulgaris*, Moyed & O'Kane (39) also observed that whereas acetyl CoA was formed in the presence of CoA, conversion to acetate and CO₂ did not require CoA. Similar findings were reported by Hager *et al.* (47). These investigators obtained a soluble pyruvate oxidase system from *E. coli* which was separated into a fraction A, active in decarboxylation of pyruvate to acetate and CO₂ when diphosphothiamine and an electron acceptor such as ferricyanide or 2,6-dichlorophenol indophenol were added. This fraction became enriched in bound lipoic acid during purification. A similar fraction, A', acted in analogous fashion on α -ketoglutarate, the products being succinate and CO₂. When a fraction, B, was added to A or A' together with diphosphothiamine, DPN⁺, CoA, and an acyl acceptor system, oxidation of the keto acid yielded acyl CoA.

These studies thus clearly distinguished two phases in pyruvate oxidation. The first is an oxidative decarboxylation requiring diphosphothiamine and lipoic acid, but not DPN⁺ or CoA. In the absence of CoA, the primary product is hydrolyzed to free acetate, but in the presence of CoA, acetyl CoA is formed and can be transferred to acceptors in the presence of the appropriate enzymes.

A present conception of the pathways involved in the decarboxylation and oxidation of pyruvic acid is shown in Figure 2, taken and slightly

⁴ The pyruvate oxidation factor, also known as α -lipoic acid or thioctic acid is the internal disulfide form of 6,8-dimercaptoöctanoic acid. Its chemistry and properties are reviewed by Cheldelin in this volume.

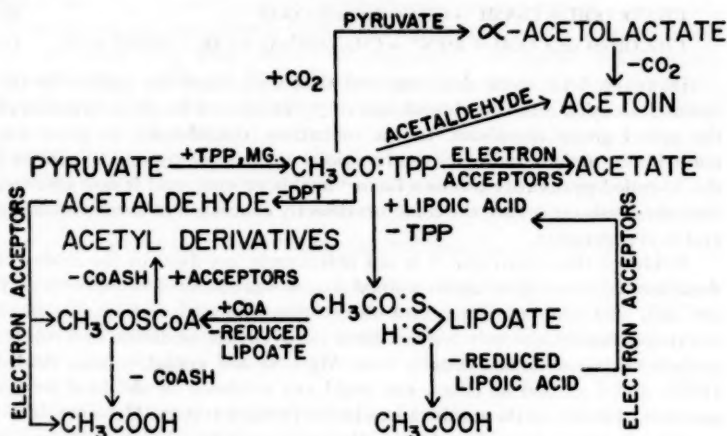


FIG. 2. Pathways in decarboxylation and oxidation of pyruvate.

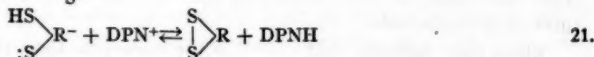
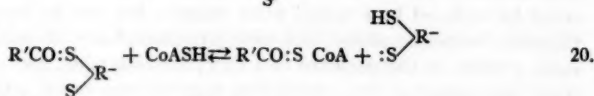
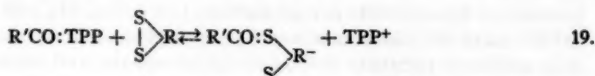
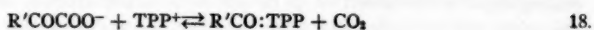
modified, from the chart by Gunsalus (35). The first step is considered to be a straight decarboxylation of pyruvate to yield an aldehyde-TPP² complex. This may undergo reaction with carbonyl compounds such as pyruvate or acetaldehyde to yield the corresponding acyloin; it may transfer electrons to an as yet unknown acceptor to yield acetate; or it may undergo reaction with α -lipoic acid to yield acetyl lipoate. Lipoic acid is apparently not obligatorily involved in the initial decarboxylation reaction, since acyloin formation can occur in *Streptococcus faecalis* and *E. coli* (47) without acyl generation, and lipoic acid is not required for this conversion. The fact that acetoin is formed from pyruvate by highly purified preparations of α -carboxylase from wheat germ (48) and yeast (49) with cocarboxylase as the only organic cofactor may also be cited in support of the nonparticipation of lipoic acid in this step. Also, Moyed & O'Kane (39) found that the pyruvate oxidase of *P. vulgaris* requires only cocarboxylase but not POF,² when coupled with an artificial electron acceptor, to yield acetate and CO₂ as reaction products. Evidence for the direct conversion of pyruvate to acetate and CO₂ in the presence of ferricyanide as electron acceptor in the animal oxidase was provided by Schweet & Cheslock (46), but it is not certain whether the ferricyanide acted on the aldehyde-TPP complex or at a subsequent stage since these fractions contained POF. That lipoic acid is not involved in the straight decarboxylation of pyruvate in *Tetrahymena pyriformis* S was shown by Seaman (50). When pyruvic oxidase from the protozoon was treated with alumina to remove POF, and incubated anaerobically with pyruvate and cocarboxylase in the presence of radioactive CO₂, incorporation into pyruvate was higher than with the untreated enzyme, or with

the treated enzyme supplemented with lipoic acid. The incorporation was attributed to reversibility of the straight, TPP-catalyzed decarboxylation reaction (equation 18).

Exchange between CO_2 and pyruvate promoted by Mg ions and co-carboxylase was also observed in the pigeon breast pyruvic oxidase by Goldberg & Sanadi (51), but the presence of lipoic acid in this enzyme makes it uncertain at what stage in the process reversibility occurred.

The reaction between the aldehyde-TPP complex and α -lipoic acid (equation 19) is visualized by Gunsalus (35) as involving a transfer of the aldehyde to one of the lipoic acid sulfhydryls, with a reductive cleavage of the S-S bond, to yield acetyl-lipoic acid. However, this step is conjectural, and further elucidation will probably have to await separation of the specific enzymes. The principal basis for this step is the observations that whereas lipoic acid is apparently not necessary for the direct conversion of pyruvate to acetate and CO_2 it is required for acetyl CoA formation. This has been demonstrated specifically by Seaman (52). The oxidation of pyruvate and α -ketoglutarate to acetate and succinate by a partially purified oxidase obtained from extracts of *T. pyriformis* S in the presence of artificial electron acceptors showed no requirements other than Mg ions. In the presence of CoA and cysteine, the preparation yielded acetyl CoA, which could be trapped with hydroxamic acid. When the oxidase was shaken with alumina, a treatment which removes lipoic acid, oxidative activity was unimpaired, but all acylative activity was lost. Complete recovery of activity was achieved by addition of synthetic 6,8-lipoic acid to the inactivated enzyme.

According to Gunsalus (35), the over-all process of pyruvate dehydrogenation, with the ultimate formation of acetyl CoA and CO_2 can be expressed in generalized form in the following four equations, in which $\text{R}'\text{COCOO}^-$ may be pyruvate, or some other keto acid, such as α -ketoglutarate, α -ketobutyrate, or others. Although, as mentioned, reactions 18 and 19 are still obscure, good evidence is now available for the specific reactions 20 and 21.



The presence of lipoic transacetylase in fractions A and A' of the pyruvic oxidase system, isolated from *E. coli* by Hager *et al.* (47), has been demonstrated in work cited by Gunsalus in his recent review (35). By coupling this

system with phosphotransacetylase and using acetyl phosphate as substrate in the presence of catalytic amounts of CoA, the reversal of reaction 20 was measured by disappearance of sulfhydryl groups or by formation of acet-hydroxamic acid. Only one of the four SH groups of D,L-lipoic acid is acetylated, indicating that only one of the four isomers is active.

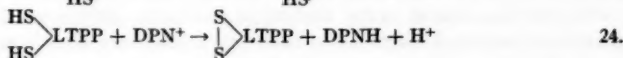
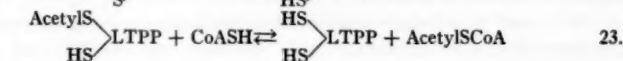
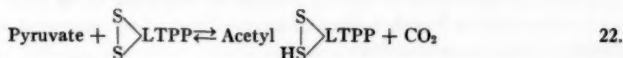
As seen in Figure 2, the operation of the pyruvic oxidase system requires a constant replenishment of lipoic acid for reaction 19. This is provided by the action of DPN⁺-specific reduced-lipoic dehydrogenase present in the B fraction of the *E. coli* oxidase. This reaction was most conveniently followed by using reduced lipoic acid as substrate and coupling its oxidation with lactic dehydrogenase. In the presence of substrate, pyruvic acid, lactic dehydrogenase, and catalytic quantities of DPN⁺, the reaction can be followed by measuring residual sulfhydryl groups.

Participation of lipothiamide pyrophosphate in pyruvate oxidation.—In a remarkably interesting series of communications, Reed & DeBusk (53, 54, 55) have presented data on pyruvate oxidation in *E. coli*, which in certain respects are at striking variance with the preceding results of Gunsalus and his group. These investigators have presented evidence that the coenzymatic form of thiamine pyrophosphate and α -lipoic acid is a compound in which the carboxyl group of the latter is attached in an amide linkage to the amino group of the former. This has been named lipothiamide pyrophosphate, LTPP. By irradiation of wild-type cells of *E. coli*, a mutant was obtained which did not grow in the presence of α -lipoic acid, but did respond to complexes of α -lipoic acid present in cell extracts of the wild-type, or to substances formed by incubating thiamine and lipoic acid with wild-type cells of *E. coli*, *Streptococcus lactis*, or *S. faecalis*, or to substances formed by purely chemical reaction between lipoic acid and thiamine or thiamine phosphate. The active principles prepared biologically by or present in extracts of wild-type cells were chemically identical with synthetic lipothiamide or lipothiamide monophosphate. Resting whole cells of the mutant required these conjugates for oxidation of pyruvate or α -ketoglutarate, but mixtures of thiamine and α -lipoic acid were inactive. Extracts of the mutant in the presence of lipothiamide pyrophosphate, L-cysteine, Mg and Mn ions, CoA, DPN⁺, lactic dehydrogenase, and CoA-phosphotransacetylase brought about dismutation of pyruvate to CO₂, acetyl phosphate, and lactate. The LTPP² could be replaced by a boiled yeast extract, but not by lipothiamide, lipothiamide monophosphate, thiamine pyrophosphate, or α -lipoic acid. The same system, in the presence of LTPP, sulfanilamide, and pigeon liver extract (the source of the acetylating enzyme) converted α -ketoglutarate to succinyl sulfanilamide.

These data indicate that LTPP is the coenzyme form of thiamine and lipoic acid and that the block in the mutant is the inability to conjugate the two. Further evidence for this view was provided by Reed & DeBusk (56) who found, in wild-type *E. coli*, a conjugase which makes LTPP from α -lipoic acid and thiamine pyrophosphate. Fractions A and B of *E. coli* were prepared

as described by Korkes *et al.* (37) from both wild and mutant strains. It was found that in the pyruvate dismutation system described above, fraction A from either strain (A_m or A_w) was active when fortified either with LTPP, or a mixture of fraction B of wild type (B_w) and thiamine pyrophosphate (TPP). However, fraction B from the mutant (B_m) was inactive with TPP in replacing LTPP. Thus fraction B appears to contain α -lipoic acid and a conjugase which synthesizes LTPP in the presence of TTP. In support of this view it was found that incubation of B_w alone with TPP produced a heat-stable product which replaced LTPP, but heating of B_w prior to incubation prevented formation of the cooxidase.

Reed & DeBusk (57, 58) presented evidence that the over-all conversion of pyruvate to acetyl CoA (equation 15) in the *E. coli* mutant may be expressed in the following three single steps.



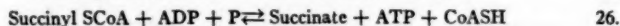
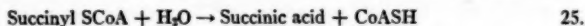
In the presence of a purified apooxidase prepared from the mutant, fortified with LTPP and Mg ions, pyruvate disappeared with the simultaneous stoichiometric appearance of CO_2 , free -SH groups, and acetylmercaptan (reaction 22). In the presence of lactic dehydrogenase, CO_2 , and DPNH, reaction 22 could be reversed, with the formation of lactic acid; and the coupled oxidation of DPNH could be followed by decrease in absorption at 340 μ . In the presence of CoA and the pigeon liver sulfanilamide acetylation system, the acetylated LTPP generated in reaction 22 was transferred to sulfanilamide. The reversibility of reactions 22 and 23 combined was demonstrated by generating acetylSCoA from acetyl phosphate and phototransacetylase, and by reducing the pyruvate thus formed to lactate by means of TPNH 2 , the reaction having been followed spectrophotometrically at 340 μ . Evidence for reaction 24 was obtained spectrophotometrically, but it could not be reversed, apparently as a result of a high negative poten-

tial for the $\begin{array}{c} \text{S} \\ | \\ \text{---} \text{LTPP} \\ | \\ \text{S} \end{array} / (\text{HS})_2\text{LTPP}$ system. TPN $^+$ was inactive in reaction 24.

Gunsalus (35) in discussing results of Reed & DeBusk has enumerated various observations which are not in accord with the idea that lipoic acid and diphosphothiamine function as a single entity. (a) According to Moyed & O'Kane (39) oxidation of pyruvate by enzymes of *P. vulgaris* require TPP but not pyruvate oxidation factor, which is apparently not present in this enzyme. (b) Though Reed & DeBusk (55) found that TPP was inactive

in pyruvate decarboxylation, in fact actually inhibiting the reaction, TPP was active with the purified apoenzyme in activating conversion of pyruvate to acetate and CO_2 , if ferricyanide was present as an electron acceptor. (c) Formation of acetoin in various bacterial systems requires TPP but not lipoic acid. Gunsalus has cited some further evidence concerning the respective functions of the *E. coli* fractions A and B, which are also not in agreement with the postulations of Reed & DeBusk on the role of LTPP: (a) during purification, lipoic acid concentrates with fraction A and not fraction B; (b) in the pyruvate oxidase of *S. faecalis*, the dissociation constant for TPP is about 100 times greater than that for lipoic acid; (c) partially purified *E. coli* A fraction can be freed of TPP and activity by dialysis against alkaline pyrophosphate and versene, and can be reactivated by adding TPP. It is unlikely that this treatment would split the amide linkage of a compound like LTPP, nor would an amide linkage be expected to be formed without an energy source. Further discussions by Reed of the role of LTPP will be found in a recent review (59).

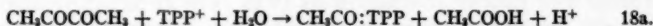
Oxidation of α -ketoglutarate.—The similarity in requirements for both pyruvate and α -ketoglutarate oxidation in *E. coli* (47, 58) and in *T. pyri-formis* (52) [see also data for *Azotobacter vinelandii* (60)] leaves little doubt that the sequence of reactions for both keto acids is the same in these organisms. That essentially the same mechanism is operative in animal tissues is indicated by studies of Sanadi & Littlefield (61) and Kaufman *et al.* (62). Both groups have described a soluble enzyme system from pig heart which in the presence of CoA, carries out the reduction of DPN^+ with the formation of CO_2 and succinyl CoA; the general course of events and the methods for assaying enzymatic activity were analogous to previous studies with pyruvate (38, 44). Sanadi & Littlefield (61) have isolated succinyl CoA and found its behavior to be quite similar to that of acetyl CoA, forming hydroxamic acid with hydroxylamine and acetylating sulfanilamide. Though the role of thiamine pyrophosphate and α -lipoic acid are not so well delineated in the animal as in the bacterial enzymes, the presence of these factors in the pig heart preparation (63) suggests that the same steps are involved in both the bacterial and animal systems. Several pathways are available for the succinyl CoA produced in the oxidation of α -ketoglutarate. Succinyl CoA deacylase, catalyzing reaction 25, has been described by Gergely *et al.* (64), and Kaufman, *et al.* (62). The second is a reversible reaction with ADP to yield succinate and free ATP, (equation 26) which is of special significance



in that it leads to the formation of a high energy phosphate bond. Properties of this enzyme have been reported by Kaufman *et al.* (62) and by Sanadi *et al.* (65). The third is the transfer of CoA from succinyl CoA to other acceptors, such as acetoacetate, already discussed (27, 28).

Metabolism of diacetyl.—Equation 18 may be generalized further to in-

clude the splitting of other types of adjacent dicarbonyl linkages, e.g., diacetyl (66). The first step in the oxidation of diacetyl to two molecules of acetate by dried cells of *S. faecalis* is a split into an aldehyde-enzyme complex and a molecule of free acetic acid (equation 18a). The next steps follow

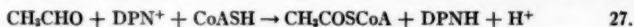


the sequence of equations 19 to 21. A similar mechanism probably is responsible for the formation of acetylmethylcarbinol and acetate from diacetyl, occurring in extracts of *Neisseria winogradski* (67). According to Juni & Heym (68) the aldehyde-enzyme complex arising by the action of several bacterial species on diacetyl can condense with diacetyl itself to yield diacetylmethylcarbinol, which in turn undergoes dismutation to acetylbutanediol, diacetyl, and acetic acid. Finally, acetylbutanediol is split to butanediol and acetic acid. Enzymes from animal tissues also can synthesize diacetylmethylcarbinol when pyruvate is oxidized in the presence of diacetyl.

Shimazu (69) has postulated a similar transketolation reaction to account for the reduction of diacetyl to acetoin which occurs in tissue "breis" in the presence of acetaldehyde. In yeast the reaction is an ordinary dismutation without cleavage of the 4-carbon chain since, in the presence of butyraldehyde, acetoin, and butyric acid are formed. In rabbit muscle "brei," however, the same reactants yield acetylpropylcarbinol and acetic acid; evidently diacetyl is split as in equation 18a to an aldehyde-enzyme complex which then condenses with butyraldehyde. The term "transcarbolygase" was proposed for the enzyme catalyzing this transfer reaction.

OXIDATION OF ACETALDEHYDE

Burton & Stadtman (70) have clarified the mechanism of the formation of acetyl phosphate from acetaldehyde in *C. kluyveri*. With extensive purification of the dehydrogenase it is now clear that the first step is a DPN⁺-linked dehydrogenation leading to the formation of acetylSCoA (equation 27), which is converted to acetyl phosphate in the presence of inorganic phosphate and transacetylase. The reaction was studied from both direc-



tions and an equilibrium constant of 1.2×10^{-4} was calculated. From this figure and thermal data from the literature, a value for the free energy of hydrolysis of acetylSCoA was computed, $\Delta F = -13,040$ cal. (pH 7.0, 25°C.). This figure is in good agreement with other values obtained in enzyme equilibrium studies, but is quite different from the value calculated by Burton & Wilson (71) from thermal data, $\Delta F = -8250$ cal. (pH 7.0, 25°C.). The enzyme also oxidized propionaldehyde, butyraldehyde, and glycolaldehyde at very low rates, but not formaldehyde, chloral, or benzaldehyde.

The authors regard the true substrate for the dehydrogenase to be a hemimercaptal of acetaldehyde and CoASH, pointing to similar suggestions

concerning dehydrogenation of α -keto acids. From considerations just discussed it appears that the aldehyde-enzyme complex arising on decarboxylation of keto acids does not react directly with CoASH but is transferred thereto after oxidation to the acetate level, presumably in a hemimercaptal linkage with thioctic acid (equations 19 to 21 or 22 to 24). It would be of interest, therefore, to learn whether thioctic acid is present in this purified aldehyde dehydrogenase. At any rate, these findings provide another instance of the general involvement of -SH compounds in the oxidation of the carbonyl group. The extensive evidence for a similar process in the dehydrogenation of triose phosphate is discussed by Anfinsen in this volume.

The possibility of a similar, direct conversion of aldehydes to acylSCoA appears questionable in animal tissues. Walkenstein & Weinhouse (72) found that in mitochondria of rat liver and kidney, acetaldehyde was oxidized quantitatively to acetic acid without the formation of acetoacetate. Since these mitochondria cannot oxidase acetate, but oxidize such acetyl CoA-forming substrates as octanoate and pyruvate to acetoacetate, it is clear that acetyl CoA could not have been the oxidation product of acetaldehyde. Higher aldehydes were oxidized to acetoacetate, but this was probably attributable to subsequent activation of the acid rather than to direct formation of acylSCoA.

Berry & Stotz (73) observed that acetaldehyde could serve as a substrate for acetylcholine and acetoin synthesis in a phosphate buffer extract of acetone dried rat cerebral cortex supplemented with ATP and CoA. Since acetate is also active in this system, acetyl CoA need not have been a direct intermediary but may have been formed subsequently from acetate, ATP, and CoA. Since the oxidation of ethanol to acetate is not impaired in pantothenate-deficient cells of *Acetobacter suboxydans* [Cheldelin *et al.* (74)] it would appear that acetyl CoA is probably not an obligatory metabolite in the oxidation of acetaldehyde by this organism. Added to the rapidly increasing number of aldehyde oxidizing enzymes is a TPN⁺-specific dehydrogenase found by Seegmiller (75) in bakers yeast. The product is acetate and no evidence for acetyl CoA formation could be obtained with the oxalacetate acceptor system.

FATTY ACID SYNTHESIS

Though it is clear that synthesis of fatty acids from acetate should occur by reversal of the individual enzymatic reactions shown in Figure 2, the net synthesis of a higher fatty acid in a purified system has not yet been demonstrated. Perhaps the closest approach was made by Brady & Gurin whose earlier findings have now been reported in detail (76). Fatty acid synthesis, determined by radioactivity assay of fatty acids isolated after incubation of tissues in the presence of C¹⁴-labeled acetate, was found to occur in whole homogenates of pigeon liver, in centrifugally separated mitochondria fortified with the soluble supernatant portion of the cytoplasm, and in water-soluble mixtures of the supernatant fluid with extracts of acetone-dried mitochon-

dria. Synthesis was stimulated by Mg ions, and by citric acid, and to a lesser extent by oxalacetic acid. The system is active in nitrogen as well as in oxygen, and is not affected by addition of insulin, growth hormone, hyperglycemic factor, α -ketoglutarate, glucose, hexose diphosphate, biotin, or dinitrophenol. Stimulation occurred with DPN⁺ and cytochrome-*c*, but not with ATP, which was inhibitory at concentrations higher than 10^{-3} M. The process was inhibited also by 0.02 M malonate, 0.1 M hydroxylamine, 0.1 M fluoride, and 0.004 M Ca ions. All activity was lost on dialysis and was not restored by addition of known factors. The water soluble system was stable to freezing and, when dialyzed lightly, displayed a requirement for DPN⁺ and Mg ions. A curious anomaly was the observation that C¹⁴-labeled acetyl CoA was less active than acetate itself in this system. A similar, though apparently somewhat more labile system was found also in rat liver by Bucher (77) and by Dituri & Gurin (78). However, a somewhat different condition exists in the rat mammary gland. According to Popjak & Tietz (79) fatty acids are synthesized in the supernatant cytoplasmic fraction of this tissue without the participation of mitochondria.

Definite evidence of the involvement of coenzyme A in lipid synthesis was provided by Klein & Lipmann (80, 81). In comparing *Saccharomyces cerevisiae* grown on pantothenate-deficient diets a correlation was found between the quantity of pantothenic acid in the growth medium, the CoA content, and the sterol yield. CoA-rich resting cells incorporated about three times as much labeled carbon of acetate into the fatty acids and sterols as did CoA-poor cells. Similarly, liver slices of pantothenic acid-deficient rats incorporated much less labeled acetate carbon into fatty acids and cholesterol than did normal tissue. Here, too, a correlation was observed between CoA content and lipid synthesis. A pantothenic acid antimetabolite, pantoyletauryl-*p*-anisidide, inhibited lipid synthesis in normal liver slices, and this could be reversed partially by pantethine but not by pantothenic acid.

STEROL SYNTHESIS

Rapid progress is being made in our understanding of the exceedingly complicated biosynthesis of the sterol ring structure. The role of acetate as a building unit has been well established for some years, and the participation of CoA in this process, already referred to (80, 81), leaves little doubt that acetyl CoA is the active intermediate in this process. Though most of the work in this field has been with intact animals or tissue slices, recent observations, using the tracer technique, of cholesterol synthesis in cell-free systems, herald the approach to an enzymatic study of the process. Although cholesterol synthesis did not occur in the cell-free systems employed by Brady & Gurin (76) for fatty acid synthesis, Bucher *et al.* (77, 82) observed incorporation of acetate carbon into cholesterol in homogenates of rat liver fortified with Mg ions and DPN⁺. The system requires oxygen, and some retention of structure is necessary, since vigorous homogenization destroyed all activity. Under optimal conditions the authors found the incorporation

to be as high as in slices. Rabinowitz & Gurin (83) prepared a water-soluble mixture of lysed rat liver particles and supernatant, which carried out the synthesis of cholesterol from acetate. The requirements were not as well defined as for fatty acid synthesis, but were presumably similar.

Intermediate formation of squalene.—Perhaps the most significant development in this field is the recognition of the intermediary participation of squalene in cholesterol synthesis. Langdon & Bloch (84) found that this highly unsaturated hydrocarbon is readily absorbed from the intestine of the rat, and 5 to 10 per cent can be recovered from the tissues. When labeled acetate was administered together with natural squalene, the recovered squalene was found to be radioactive. Its metabolic turnover is very rapid, acetate carbon having been maximally incorporated in 30 min. These results, in conjunction with the fact that only traces are normally present in rat tissues, indicate that squalene is an intermediate in a rapid metabolic process. When the labeled, biosynthetic squalene was administered to mice, radioactive cholesterol was isolated from the tissues, but isolated fatty acids were inactive (85). It was also found that the presence of squalene greatly depressed the incorporation of labeled acetate into cholesterol but not into fatty acids. Synthetic preparations of squalene containing C^{14} or deuterium, failed to yield labeled cholesterol; evidently these substances were not identical with the natural product despite their close resemblance in physical properties. Tomkins *et al.* (86) also failed to observe cholesterol synthesis from a synthetic C^{14} -labeled squalene.

Wüersch, Huang & Bloch (87) proposed, on the basis of their extensive degradation of the cholesterol side-chain, that the structural unit in cholesterol is the isoprenoid unit, and Woodward & Bloch (88) have shown how squalene, built up from such units, may be cyclized by a mechanism somewhat similar to one suggested by Robinson (89) many years ago. Extensive but still incomplete degradations of labeled biosynthetic cholesterol by Cornforth *et al.* (90) and by Dauben *et al.* (91) are in accord with the intermediary cyclization of squalene in the manner suggested by Woodward & Bloch. From similar experiments Dauben *et al.* (91) concluded that the distribution of labeled acetate carbon in the quaternary methyl carbons of biosynthetic cholesterol is also indicative of this mechanism of cyclization of squalene.

HORMONAL AND OTHER FACTORS INFLUENCING LIPOGENESIS

The synthesis of fatty acids and cholesterol is affected markedly by hormonal and dietary factors; indeed these processes represent perhaps the most striking of the few instances known in which effects of hormones on biochemical reactions can be observed *in vitro*. The hope that such studies might shed some light on the enzymatic action of hormones has stimulated much interest in lipogenesis in surviving tissue slices, particularly of liver. Thus far, the multiplicity of effects observed under various experimental conditions has rendered extremely difficult any rational interpretation of the

observations; hence the reviewer can do little beyond recording and summarizing the published data.

Lipogenesis in liver slices.—Medes, Spirter & Weinhouse (92) have pointed out that the magnitude of incorporation of acetate carbon in tissue slices does not necessarily reflect the rate of lipid synthesis and have described a procedure for determination of actual rates of lipogenesis in isolated liver slices. This involves a determination of the specific activity of the acetoacetate produced during incubation of the tissue with labeled acetate. On the assumption that the specific activity of the acetoacetate represents that of the acetyl groups undergoing condensation to cholesterol and fatty acids, rates of synthesis of these lipids were calculated. Half-times for fatty acid synthesis in liver slices were found to be considerably higher, of the order of four to nine days than were previously reported in intact rats, of the order of one to two days; this probably indicates that the conditions *in vitro* were not optimal for fatty acid synthesis. However, half-time values for cholesterol of about six days agreed with previous data with intact rats. In liver slices of both fed and fasted rats, parallel responses were observed between substrate concentration and conversion to fatty acids, cholesterol, acetoacetate, and CO_2 . The same observations were made with acetate, lactate or glucose as substrate, despite large differences in the relative conversions of each substrate. These findings were regarded as indicating that the same acetyl group, presumably acetyl CoA, is derived from all acetyl precursors, and that the marked difference, as for example between glucose and acetate in their ability to yield carbon for fatty acid formation, is attributable primarily to differences in the rates at which they yield active acetyl groups.

Wyshak & Chaikoff (93) observed that conversion of glucose to CO_2 , glycogen, and fatty acids were all impaired in fasting, but conversion of fructose to CO_2 and glycogen was not impaired, despite the fact that its conversion to fatty acids was diminished to the same extent as was that of glucose. The differences between the two sugars in their conversion to CO_2 and glycogen clearly indicated a block in glucokinase in fasting. However, the impairment of lipogenesis from fructose under conditions in which it is readily oxidized, points to an effect of fasting on lipogenesis which is distinct from the impairment in glucokinase activity.

Previous observations of marked impairment of fatty acid synthesis in liver slices of fasted rats were extended to the whole rat by Coniglio *et al.* (94), and the same behavior in liver slices of alloxan-diabetic rats has been confirmed in the intact rat by Van Bruggen *et al.* (95). However, cholesterol synthesis was not affected. Hotta & Chaikoff (96) found cholesterol synthesis to be actually above normal in liver slices from alloxan diabetic rats, a finding of considerable significance from the clinical standpoint. In keeping with the often-observed antagonism between pancreatic and pituitary function is the observation of Tomkins *et al.* (97) that cholesterol synthesis from acetate is greatly reduced in liver slices from hypophysectomized rats.

A remarkable effect of dietary cholesterol on cholesterol synthesis has been observed. Tomkins *et al.* (98) found that cholesterol in amounts of 5 per cent of the diet almost completely abolished incorporation of acetate carbon into cholesterol of liver slices. The effect was noted whether the diet was high or low in fat, and it was not accompanied by diminution in oxidation of acetate or its incorporation into fatty acids. A less pronounced effect was noted at a dietary cholesterol level of 0.5 per cent. The authors point out that in view of the enhanced synthesis of cholesterol with diets low in this substance there appears to be no advantage in attempting to control the blood cholesterol level by dietary means, a common practice in the medical management of atherosclerosis or hypercholesterolemia. Essentially similar results have been noted by Gould *et al.* (99) in tissues of dogs. Liver slices of dogs fed a diet containing 1 per cent of cholesterol showed a greatly lowered uptake of acetate carbon in cholesterol but no effect on its incorporation into fatty acids. Cholesterol synthesis was also observed in intestinal mucosa, adrenal cortex, and skin, but its rates were much lower in these tissues than in liver and were not affected by dietary cholesterol.

The effect of dietary cholesterol in inhibiting cholesterol synthesis from acetate has been confirmed by Langdon & Bloch (100), and this feature was utilized by them in the screening of possible intermediates in cholesterol synthesis. They found that cholesterol itself, squalene, Δ -7 cholesterol, and 7-dehydrocholesterol caused a marked reduction in cholesterol synthesis from acetate when these substances were fed to rats together with C^{14} -labeled acetate. A number of other sterols and terpenes, for example, lanosterol, coprosterol, ergosterol, farnesol, or an isomer of squalene, had no effect on cholesterol synthesis. The implication of these results is that those substances inhibiting cholesterol synthesis may be regarded as intermediates on the biosynthetic pathway. In a search for substances inhibitory to cholesterol synthesis Tomkins *et al.* (101), using the rat liver slices with C^{14} -labeled acetate as the test system, found that of a large group of steroids fed, four displayed marked anticholesterogenic action, viz., cholestanone, dehydroisandrosterone, 7-dehydrocholesterol, and Δ -7-cholestenol.

The reasonable conclusion that cholesterol synthesis is preceded by formation of other steroids is supported by the findings of Schwenk & Wertheissen (102). These investigators found that cholesterol synthesized by perfused swine liver from C^{14} -labeled acetate was accompanied by digitonin-precipitable material having a much higher specific activity than cholesterol, which could be removed therefrom by purification via the dibromide. These investigators also made the surprising finding that damage to the perfused liver resulted in enhancement rather than impairment in cholesterogenic activity (103).

Curran (104) has reported that cholesterol synthesis by rat liver slices was considerably greater in the pH range of 6.2 to 6.8 than in the range of 7.2 to 7.4. The suggestion was made that reduction of intracellular pH may be a significant factor in the hypercholesterolemia occurring in diseases asso-

ciated with disturbances in acid-base balance. Becker *et al.* (105) reported that scorbutic guinea pigs given acetate-1-C¹⁴ prior to sacrifice had six times higher specificity activity in the cholesterol of the adrenal glands than normal animals and also observed a higher specific activity in liver cholesterol. Whether this interesting observation is attributable to differences in the synthetic capacity of these tissues, as the authors suggest, or whether it is a result of differences in the amounts of cholesterol in the organs, or in differences in the rate of transport to and from these organs, it is not possible to determine from the condensed summary presented in their preliminary report. Spirtes *et al.* (106, 107) found that fatty acid and cholesterol synthesis proceeded at least as rapidly in liver slices of hyperthyroid rats as in those of normal, fed animals; similar findings with regard to cholesterol synthesis have been made by Byers *et al.* (108) in experiments with intact rats.

Lipogenesis in nonhepatic tissues.—In continuation of their extensive studies on milk-fat synthesis, Popjak *et al.* (109) found in confirmation of previous results, that a major portion of the short chain fatty acids of milk fat of the lactating rabbit arises by synthesis from acetyl groups in the mammary gland. The distribution of C¹⁴ activity in octanoic acid of the milk-fat formed in the presence of various labeled precursors, including acetate, pyruvate, and glucose, were entirely in accord with present conceptions of glucose breakdown and fatty acid synthesis. Balmain *et al.* (110) in a preliminary report, found marked differences in the extents of utilization of glucose and acetate for fatty acid synthesis in rat and sheep mammary gland slices, though both substrates were utilized. In a previous study these investigators found that both insulin and glycerol stimulated incorporation of acetate carbon into sheep mammary gland acids, from which it was concluded that insulin increased the availability of glycerol for glyceride synthesis. The present study with rat slices showed, however, that the effect of glycerol differs from that of insulin; though glycerol increased the utilization of acetate significantly more than glucose, insulin stimulated the utilization of both substrates equally. Kleiber *et al.* (111) have shown that propionate which is a product of cellulose digestion in ruminants can be utilized for milk fat formation in the sheep, though its main route of metabolism appeared to be directed toward carbohydrate, as indicated by the appearance of propionate C¹⁴ in the milk lactose. Though previous evidence concerning the ability of the chick embryo to synthesize cholesterol has been contradictory, Stokes *et al.* (112) have now shown that acetate-C¹⁴ is continuously incorporated into cholesterol of the hen's egg during the entire incubation period, though the rate is higher in the later stages. Coleman & Nord (113) have made further studies of fatty acid synthesis in *Fusarium lini* Bolley.

THE CITRIC ACID CYCLE

Occurrence in microorganisms.—The citric acid cycle is now generally recognized to constitute the major if not the sole mechanism for oxidation of acetyl groups in cells of higher animals and plants. The scope of this proc-

ess in microbial metabolism, though still a subject of some uncertainty, has been widely extended recently with the recognition that the inability of certain organisms to metabolize citric acid or other components of the citric acid cycle, is a result of impermeability of the cells rather than to absence of the enzymes. In many investigations, it was found that whereas whole cells are inert to one or more cycle components, these were readily formed or utilized by broken cells or extracts. This was true for *Propionibacterium pentosaceum*, strain E214 [Delwiche *et al.* (114)], *Pasteurella pestis*, strain A1122 [Englesberg *et al.* (115)], normal *S. cerevisiae* and a small colony mutant of this organism [Hirsch (116)], *Spirillum serpens* [Mather (117)], *Azotobacter agile* [Repaske & Wilson (118)], *Corynebacterium creatinovorans* [Fukui & Vandemark (119)], *Rhodospirillum rubrum* [Eisenberg (120)], *E. coli* [Grunberg-Manago & Gunasius (121)], and *Streptomyces coelicolor* [Cochrane & Peck (122)]. In two instances (115, 116), citric acid cycle enzymes were found to be under adaptive control, being low or absent in anaerobic-grown, but present in aerobic-grown cells. Other organisms in which enzymes of the cycle were present or in which citric acid accumulated under certain conditions are: *Neisseria gonorrhoeae* [Tonhazy & Pelczar (123)], *Achromobacter guttatus* [Sgueros & Hartsell (124)], *Pseudomonas fluorescens* [Barrett & Kallio (125)], *Mycobacterium phlei* [Blakley (126)], *Ashbya gossypii* [Mickelson & Schuler (127)], *Streptomyces griseus* [Coty *et al.* (128)], and *Penicillium Chrysogenum* [Goldschmidt & Koffler (129); Miller *et al.* (130)].

The study of Wang *et al.* (131) is of especial interest for its quantitative implications. It was found that baker's yeast, grown in the presence of carboxyl-labeled acetate, yielded glutamic acid with activity in the carboxyl carbons, but with twice as much activity in the γ as in the α -carboxyl. This is precisely the ratio to be expected if glutamate is derived from α -ketoglutarate formed by extensive operation of the cycle. If the cycle stopped at α -ketoglutarate the glutamate would be labeled only in the γ -carboxyl. The extent of labeling in the α -carboxyl indicates that essentially all of the acetate reacted with "recycled" oxalacetate; such extensive recycling shows that the cycle represents a major oxidative pathway of metabolism rather than just a means of synthesis of glutamic acid.

Some evidence for other pathways has been offered. Goucher *et al.* (132) found that dinitrophenol inhibited succinate oxidation almost completely in cells of *E. coli*, strain B, at levels which only slightly inhibited acetate oxidation. The authors concluded that in the presence of dinitrophenol, acetate is oxidized by a pathway not involving di- or tricarboxylic acids. Cabelli & Baumstark (133) concluded that *Klebsiella pneumoniae* do not carry out reactions of the cycle, based on the inactivity of citrate and related acids. Eaton & Klein (134) found that cells of *S. cerevisiae*, harvested during the log phase of growth (young cells), oxidized acetate at a lower rate than glucose, whereas "old" cells oxidized both at equally high rates. The suggestion was made that "young" cells have an impaired citric acid cycle, but

the basis for this suggestion is not clear from the data presented in the abstract. It is now evident that experiments with whole cells do not necessarily provide reliable criteria for their enzymatic capabilities. These studies would have been more convincing if they were supplemented by data on preparations other than whole cells. The possible occurrence of a dicarboxylic acid cycle is still under discussion, but indisputable proof of such a process still awaits discovery of an enzyme which directly converts acetate to a 4-carbon dicarboxylic acid.

To the reviewer's knowledge, the only aerobic organism in which absence of the citric acid cycle has been convincingly shown is *Acetobacter suboxydans*. King & Cheldelin (135) found that oxidation of pyruvate and lactate by three separate strains of this organism gave quantitative yields of acetate and CO_2 . Intact cells and cell-free preparations were completely inert to acetate and members of the citric acid cycle, though they readily oxidized ethanol, glycerol, dihydroxyacetone, sorbitol, and glucose. Though bound forms of pantothenic acid, including coenzyme A, are present, indicating participation of coenzyme A in the metabolism of this organism, acetylation of sulfanilamide, or oxalacetate did not occur.

Studies of individual citric acid cycle reactions.—Though no major changes in the cycle have developed, considerable progress has been recorded in our understanding of some of the individual stages, and some interesting variants and extensions have been reported. On the basis of kinetic data, Friedrich-Freska & Martius (136) proposed that the enzyme, aconitase, catalyzed the direct interconversions of citrate, isocitrate, and *cis*-aconitate; that is, citrate and isocitrate may be interconverted without formation of *cis*-aconitate, which was regarded as a by-product. The principal basis for this view was the absence of a lag in the conversion of citrate to isocitrate such as would occur during the initial stage of the reaction while the *cis*-aconitate concentration was being built up to its optimum. In a detailed analysis of the kinetics of aconitase action Kacser (137) pointed out that the calculations of Friedrich-Freska & Martius were based on several unreliable values and assumptions; his own data indicated that *cis*-aconitate is a necessary intermediate in the interconversion of citrate and isocitrate. Further evidence of the participation of *cis*-aconitate in the aconitase reaction was provided by Krebs & Holzach (138). Using a dilute suspension of heart muscle as a source of aconitase, the conversion of *cis*-aconitate to isocitrate proceeded with maximal velocity and tapered off gradually, whereas the conversion of citrate to isocitrate displayed a distinct lag, hardly any isocitrate being produced in the first five minutes of reaction.

A "citrate oxidation factor," active in causing the disappearance of citrate in dialyzed extracts of *S. cerevisiae* has been concentrated by Foulkes (139). It has not been characterized chemically, but the properties do not correspond with any known factors. It is heat-stable, weakly basic, and contains no phosphate. The reaction which is accelerated by this substance is uncertain. It was originally thought that the product was α -ketoglutarate,

formed by a pathway different from the conventional one; however, it now appears that the product is not an α or β keto acid, nor is it lactic or a volatile acid. Unlike the conventional pathway of citrate oxidation, this one requires oxygen as an electron acceptor and is cyanide sensitive (140). The nature of this process is totally obscure at present.

An unusual type of citric acid fermentation was found in *S. faecalis*, strain 10C1, by Gillespie & Gunsalus (141). Extracts of this organism cleave citrate into acetate and oxalacetate; the latter accumulates as such when the reaction is carried out in extracts freed of oxalacetic carboxylase. The reaction requires Mn ions but not CoA, and though the extracts contain a CoA-dependent transacetylase they are free of acetyl phosphate or acetyl CoA hydrolases. Thus it appears that acetyl CoA is not an intermediate, and the reaction is therefore not a reversal of the condensing reaction. The enzyme catalyzing the cleavage was termed "citridismolase."

A similar or identical system was found in *Aerobacter aerogenes* by Dagley & Dawes (142). In cell-free extracts of this organism, citrate was converted to one molecule each of acetate and pyruvate; the latter was derived presumably from oxalacetate, which could be detected when the decarboxylase was inhibited. The process was not a reversal of the condensation reaction; it was adaptive and suppressed by aerobic growth; and there was no evidence of CoA-involvement. Srere & Lipmann (143) have described another enzyme in pigeon liver, apparently different from and separable from Ochoa's condensing enzyme, which, in the presence of ATP and CoA, catalyzes the reversible transformation of citrate to oxalacetate, acetyl CoA, ADP, and inorganic phosphate, according to equation 28.

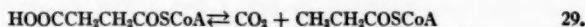


It would appear that the first step in the forward reaction is an ATP-promoted esterification of citrate, but the authors were unable to show the formation of citroyl CoA. This enzyme differs from the Ochoa condensing enzyme in that with the latter, citrate and CoA react directly to yield acetyl CoA and oxalacetate without the intervention of ATP.

A new deviation from the cycle has been discovered by Campbell *et al.* (144) in *Pseudomonas aeruginosa*. "Sonic extracts" of this organism did not yield α -ketoglutarate from citrate under aerobic conditions but carried out a split into succinate and glyoxylate; this transformation also occurred anaerobically. A puzzling feature is that whereas the conversion was observed with either citrate or *cis*-aconitate, isocitrate was inactive and was not converted to citrate. Either aconitase was not present or was inactivated in the extract. This reaction is of considerable interest in indicating new possible routes of synthesis of glycine and oxalic acid.

Decarboxylation of succinate.—Whitely (145) has shown, in studies with cell-free extracts of *Micrococcus lactilyticus*, strain 221, that the decarboxylation of succinate to yield CO_2 and propionic acid requires ATP, cocarboxylase, and coenzyme A. The probable participation of succinyl CoA as an

intermediate was indicated by the formation of succinhydroxamic acid in the presence of hydroxylamine and by the acylation of sulfanilamide by the same preparation. Further evidence of the participation of succinyl CoA in the decarboxylation was provided in an isotope tracer study by Phares *et al.* (146). When 4-C¹⁴-succinyl-1-CoA was incubated with cell-free extracts of *Propionibacterium pentosaceum*, the radioactivity of the CO₂ was the same as when 1,4-C¹⁴-succinyl-1-CoA was used as a substrate. However, the propionate from the symmetrically labeled succinyl CoA had six times the activity of the propionate derived from the asymmetrically labeled ester. The data suggest that the reaction occurs as indicated in equation 29 to yield CO₂ and propionyl CoA.



Since propionic acid was incorporated into succinate at 50 to 75 times the rate of CO₂ incorporation (147), it was suggested that succinate decarboxylation yields a one-carbon compound which can reversibly combine with propionyl CoA and does so in preference to its conversion to CO₂. That at least part of the propionate formed by cellulose-utilizing microorganisms of the sheep rumen is derived from succinate is suggested by results of Sijpesteijn & Elsdén (148). Wood & Leaver (149, 150) pointed out, from results on carbon balances and isotope-labeling, that certain features of the formation of propionate by the propionic acid bacteria are not in complete accord with established metabolic pathways.

The possibility that a variant of the citric acid cycle may exist in which acetate and α -ketoglutarate condense to give a 7-carbon analogue of citric acid was envisioned by Strassman & Weinhouse (151). The distribution of C¹⁴ in samples of lysine obtained from *Torulopsis utilis* grown in the presence of C¹⁴-labeled acetate was in accord with a mechanism embracing the following steps: formation of a 7-carbon "homocitrate" by condensation of acetate with α -ketoglutarate, conversion to "homoisocitrate," and oxidation and decarboxylation to α -ketoadipate, a known precursor of lysine in *Neurospora*.

Condensation of succinate with glycine.—A long step forward has been taken by Shemin & Russell (152) in our understanding of the role of succinate in porphyrin synthesis. In confirmation of the previous suggestion that succinate condenses with the α -carbon of glycine, these investigators have now found that γ -aminolevulinic acid, a decarboxylation product of α -amino- β -ketoadipic acid, the postulated condensation product of glycine and succinate, is a close precursor of the porphyrin ring. Presumably two molecules condense to give a carboxylated protoporphyrin. The authors suggest that these substances may also be involved in purine and serine synthesis from glycine.

Thermodynamic data.—An impressive series of papers containing thermodynamic data on reactions of the citric acid cycle has appeared from the laboratory of H. A. Krebs. In a study of aconitase and fumarase equilibria

it was found that at pH 7.5 and 25°C. the fumarase system contained 81.55 per cent malate and 18.45 fumarate (ratio = 4.42) and the aconitase system contained 6.20 per cent isocitrate, 2.90 per cent *cis*-aconitase, and 90.9 per cent citrate (153). Burton & Wilson (71), by measuring equilibrium concentrations of products and reactants in the alcohol dehydrogenase-catalyzed reduction of DPN⁺ by isopropanol, and combining these data with available thermal data for the free energy of formation of isopropanol and acetone, calculated a value of 5.22 kcal. (-0.320 v.) for the free energy (ΔG°) of reduction of DPN⁺. Combining this value with measurements of equilibrium concentrations of DPN⁺ and DPNH in various dehydrogenase reactions, $\Delta G'$ values for pH 7 were calculated for the following: ethanol to acetaldehyde, 9.68; isopropanol to acetone, 5.89; L-lactate to pyruvate, 10.32; L-malate to oxalacetate, 11.45; L-glycero-1-phosphate to dihydroxyacetone phosphate, 10.24; glyceraldehyde-3-phosphate to glyceroylphosphate-3-phosphate, 5.83; (+) isocitrate to oxalosuccinate, 3.74; and (+) isocitrate to α -ketoglutarate, -3.14 kcal. $\Delta G'$ value for DPNH to DPN⁺ was 4.33 and for TPNH to TPN⁺ was 4.11 kcal.

With the aid of these data and utilizing, where available, other thermochemical data from the literature, Burton & Krebs (154) calculated accurate values for the free energy of formation of various key substances associated with reactions of the citric acid cycle. These were then used in calculating the free energies of individual steps of the cycle, glycolysis alcoholic fermentation, hydrolysis of the pyrophosphate group of ATP, and various associated reactions. Values are given for ΔG° (standard state) and ΔG , the "physiological state," namely, 0.2 atm. O₂, 0.05 atm. CO₂, pH 7 and 0.01 *M* concentration of other reactants.

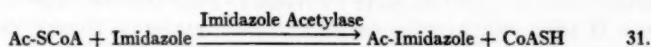
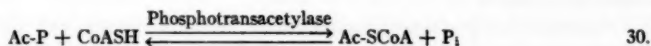
Peters *et al.* (155) have isolated the long-postulated monofluorocitric acid which is formed enzymatically in the condensing reaction from fluoroacetate and oxalacetate and have described its chemical properties. Comparison of its infrared spectrum with that of a synthetic product (156) has provided final proof of its identity. The synthetic material has one-half of the inhibiting action on aconitase of the natural product, indicating that only one of the two asymmetric centers is concerned with its inhibitor action.

BIOLOGICAL ACETYLATION

Disclosure of the role of CoA in acetylations has stimulated considerable interest in this process. Katz *et al.* (157) have supplied further details of the acetylation of amino acids by dried cells of *C. kluyveri*. All of the common amino acids were acetylated by acetyl phosphate, as were several peptides and aliphatic amines. A peculiar property of this system is the pronounced activation by cyanide; this effect was also given by azide, but not by other reducing agents, carbonyl group reactants, or metal binders. Certain common properties suggested that the reaction is catalyzed by the phosphotransacetylase of this organism, the actual acetylating agent being acetyl CoA. The authors provided further evidence for this view in an extension of this

study to other acids. It was found that propionyl phosphate, which is active in the phosphotransacetylase reaction and can also serve as a substrate for fatty acid chain elongation in this organism, propionylated amino acids, whereas *n*-butyryl phosphate was inactive in all three reactions unless "activated" by the addition of acetyl or propionyl phosphate. This "activation" apparently consists in an exchange of CoA between the acetyl or propionyl group and the *n*-butyryl group. The conversion of acyl phosphate to acyl CoA thus appears to be a common initial step in all three reactions.

Stadtman & White (158) have discovered a CoA-dependent acetylation of imidazole in an extract of *C. kluyveri*, which is of unusual interest in that the product appears to have a high free energy of hydrolysis; in aqueous solution it reacts spontaneously with amino acids, alcohols, inorganic phosphate, sulfhydryl compounds, and hydroxylamine, to form the corresponding acetyl derivatives. The enzymatic acetylation of imidazole by acetyl phosphate in a partially purified preparation requires phosphotransacetylase and CoA; thus, the probable course of events is as shown in equations 30 and 31.



The authors suggest that imidazole may be a model for the physiological transfer of acetyl groups, which might involve imidazole moieties of proteins or coenzymes as acyl carriers.

Tabor *et al.* (159) described the properties of a pigeon liver acetylating enzyme, finding that it acetylates a wide variety (but not all) of aromatic amino acids, histamine, and, to a small extent, glucosamine. A convenient acceptor for the acetyl group of acetyl SCoA was *p*-nitroaniline, the acetylation of which could be followed by a change in its absorption spectrum in the visible region. According to Chaveau & Hung (160) this enzyme is localized in the soluble, homogeneous cytoplasmic phase of the pigeon liver cell. Proof that benzoyl CoA is the active intermediary in hippurate formation, previously indicated by the dependency of this reaction on ATP and CoA, has been provided by Schachter & Taggart (161). As previously described for acetyl CoA the S-benzoyl ester can transfer its benzoyl group nonenzymatically to other sulfhydryl compounds.

Hansenula anomala carries out a large scale acetylation of ethanol; under optimal aerobiosis on glucose, it can produce up to 30 per cent yields of ethyl acetate. This substance is not an end-product, but accumulates as a transient intermediate, which is oxidized rapidly on exhaustion of the substrate (162, 163). Some remarkable acetylations have been reported by Bolcato *et al.* During the oxidation of acetate by baker's yeast in the presence of phenylhydrazine, the phenylhydrazide of acetic acid was produced (164). The same product was obtained in similar experiments with *A. niger* but not with

E. coli (165). It was also formed during the oxidation of D-xylose by *T. utilis* (166). Even more remarkable was the formation of acetophenone when acetate-grown *E. coli* was allowed to respire in the presence of sodium acetate and phenylhydrazine oxalate (167). This reaction, which in effect, represents an acetylation of the benzene ring, has now been found to occur non-enzymatically (168) with phenylhydrazine oxalate and acetaldehyde (conditions not stated).

SOME ASPECTS OF CARBOHYDRATE METABOLISM

NEWER PATTERNS OF CARBOHYDRATE DISSIMILATION

General recognition that oxidative pathways of glucose catabolism proceed via 6-phosphogluconic acid and pentose phosphates has stimulated considerable interest in the metabolism of postulated intermediates of such pathways. The realization of the role of "active glycolaldehyde" in certain of these transformations and the discovery of transaldolase and transketolases represent probably the most striking of the new enzymatic developments in this field.

Transaldolases and transketolases.—It is becoming increasingly clear that many synthetic reactions occur by transfer rather than by direct combination of substrates, as examples may be cited numerous recently-described transphosphorylations, transacetylations, etc. In line with this modern trend is the extension of this concept to reactions involving the carbon-to-carbon linkage. Independent evidence for this type of reaction in the formation of several keto-sugars has been advanced from two laboratories. In a study of the mechanism of formation of sedoheptulose phosphate from pentose phosphate, Horecker & Smyrniotis (169) found that a highly purified enzyme from spinach catalyzed the formation of ketoheptulose phosphate from L-erythrulose and D-glyceraldehyde-3-phosphate. Since this enzyme also splits ribulose-5-phosphate into triose phosphate and an as yet unidentified "diose," presumably glycolaldehyde, two possibilities were envisioned for heptose formation, viz., combination of two dioses to yield erythrulose, which in turn could condense with D-glyceraldehyde-3-phosphate to yield the heptulose phosphate; or, combination of the "diose" with ribose-5-phosphate. The latter could have been formed from ribulose-5-phosphate because pentose isomerase was present in the enzyme preparation. In any case, the reaction requires the participation of glycolaldehyde, and since glycolaldehyde itself is neither demonstrable nor active in this system it would appear that the glycolaldehyde is transferred without dissociation from an enzyme complex.

In a parallel investigation Racker *et al.* (170) isolated a crystalline enzyme from yeast which catalyzes the cleavage of ribulose-5-phosphate to D-glyceraldehyde-3-phosphate, but only in the presence of an acceptor aldehyde, such as ribose-5-phosphate, glycolaldehyde, or glyceraldehyde. The nature of the products formed were not stated, but they were, presumably, the

corresponding keto sugar formed by condensation of the carbonyl carbon of the acceptor aldehyde with that of glycolaldehyde. Evidence for this formulation was obtained using β -hydroxypyruvate as substrate. In presence of D (or DL) glyceraldehyde-3-phosphate as acceptor, ribulose-5-phosphate was formed. Apparently the enzyme catalyzes the decarboxylation of hydroxypyruvate to yield a glycolaldehyde complex, whose carbonyl group condenses with that of the aldehyde to yield the ketopentose phosphate. Racker (171) has pointed out the resemblance of this process to acetoin synthesis, in support of which is the requirement of thiamine pyrophosphate, noted by both groups (169, 170). Since the enzyme catalyzes the cleavage and formation of α -ketols, it has been named transketolase. It is interesting to note that somewhat similar types of reactions were postulated by Akabori *et al.* (172). Dihydroxymaleic acid was found to be decarboxylated to hydroxypyruvic acid and to yield erythrose when incubated with minced rabbit muscle. The same preparation isomerized erythrose to a nonreducible product, presumably erythrulose, and when dihydroxymaleic acid and erythrose were incubated together, fructose and glucose were detected as reaction products. These investigators postulated a step-wise condensation of glycolaldehyde moieties arising by decarboxylation of hydroxypyruvate, to yield successively, erythrulose, erythrose, fructose, and glucose. In support of this mechanism, it was reported that when fructose-1,6-diphosphate, aldolase, and dihydroxymaleic acid were incubated with the rabbit muscle mince, ribose was isolated; the suggestion was offered that the pentose arose by addition of a glycolaldehyde moiety to triose phosphate generated *in situ*. However, glycolaldehyde did not replace dihydroxymaleic acid in this system.

The similarity of the transketolation reaction to other reactions involving the formation and cleavage of carbon-to-carbon bonds, such as the decarboxylation of α -keto acids, cleavage of diacetyl, acyloin formation, etc. has been pointed out by several investigators. A generalized concept may be embodied in equations 32 and 33.



RCHO may be acetaldehyde derived from pyruvate or diacetyl; succinic semi-aldehyde derived from α -ketoglutarate; or glycolaldehyde derived from hydroxypyruvate, ribulose-5-phosphate, or other donor molecules; in each instance, the aldehyde-enzyme complex reacts with an acceptor, which as we have seen, may be lipoic acid, an aldose, or other aldehyde, or possibly even CO_2 . The participation of thiamine pyrophosphate as a coenzyme in all of these transformations is a further reflection of their fundamental similarity in mechanism.

Another similar type of reaction was described by Horecker & Smyrniotis (173). These investigators demonstrated, with enzymes from yeast, a trans-

fer of a triose moiety from sedoheptulose-7-phosphate to D-glyceraldehyde-3-phosphate to yield fructose-6-phosphate. When the reaction was conducted with C^{14} -labeled triose phosphate, the fructose had activity only in carbons 4, 5, and 6, and none in carbons 1, 2, and 3. Thus, the hexose could not have been formed by the ordinary aldolase reaction which would have randomized and doubled the radioactivity; hence carbons 1 to 3 could only have come from the heptulose carbon chain. It was concluded that the enzyme catalyzed the transfer of carbons 1 to 3 of the heptulose (the dihydroxyacetone moiety) to triose phosphate. The tetrose ester thus formed from carbons 4 to 7 was not identified, but after hydrolysis with phosphatase it gave a spot identical in R_f with erythrose. This reaction differs from the transketolation just discussed in that the split occurs between two adjacent hydroxyl groups rather than between adjacent carbonyl and hydroxyl carbons; thus the reaction is analogous to the aldol rather than the ketol condensation. Accordingly the enzyme has been termed transaldolase. The authors suggest that many sugar transformations may occur by means of analogous reactions. The often-observed conversions of pentoses to hexoses, particularly, those transformations to be discussed in the next section which involve the intermediary formation of sedoheptulose phosphate, observed in spinach leaf extracts by Axelrod *et al.* (174), and in liver by Glock (175), probably occur by this or a similar mechanism.

A novel aldolase type of reaction has been reported by Charalampous & Mueller (176) consisting in the condensation of formaldehyde with triose phosphate (presumably dihydroxyacetone phosphate) to yield L-erythrulose-1-phosphate. Using C^{14} -labeled formaldehyde, the identity of the product was established by combined paper chromatography and chemical degradation. The enzyme responsible for this reaction is not the classical aldolase. Another condensation involving formaldehyde has been reported by Hift & Mahler (177). An enzyme obtained from beef liver mitochondria catalyzes the condensation of formaldehyde and pyruvate to yield α -keto- γ -hydroxybutyric acid. A similar reaction with β -phenylpyruvic acid yielded the corresponding aromatic acid, but other keto acids were inactive.

Pathways of hexose and pentose metabolism.—It is now evident that oxidative and fermentative pathways of glucose metabolism, proceeding via hexose monophosphate and 6-phosphogluconate are far more prevalent than was generally believed.^a The best established of these is the so-called shunt, which involves the participation of phosphorylated pentoses, and which may

^a The reviewer wishes to point out that the designation of the Embden-Meyerhof process as the "glycolytic," and others as "nonglycolytic" is incorrect. The term "glycolysis" should be used to indicate glucose disappearance, or in the sense used by Warburg, the production of lactic acid from glucose. It should not carry any implication of pathway. It should also be pointed out that the term "oxidative pathway" or "oxidative shunt" does not indicate the necessary involvement of oxygen. No differentiation between glucose dissimilation pathways can be made as yet on the basis of aerobiosis or anaerobiosis.

be represented as follows: glucose \rightarrow glucose-6-P \rightarrow 6-P-gluconic \rightarrow CO₂ + ribose-5-P \rightarrow ribulose-5-P \rightarrow triose P + "diolose."

Information on the occurrence of the shunt pathway in plants was provided by several studies. Axelrod *et al.* (174) demonstrated the presence of dehydrogenases for glucose-6-phosphate and 6-phosphogluconate in spinach leaf extract. When acting on ribose-5-phosphate or ribulose-5-phosphate, this preparation yielded triose phosphate (mainly dihydroxyacetone phosphate) and sedoheptulose phosphate. The latter was further metabolized to hexose phosphates which yielded glucose and fructose on hydrolysis. That these reactions may be widespread in plants is indicated by studies of Barnett *et al.* (178), who demonstrated the presence of the TPN-specific 6-phosphogluconic dehydrogenase, with the formation of CO₂ and pentose phosphate, in a variety of plant tissues. A preparation from parsley leaves converted ribose-5-phosphate to ketoheptose. Enzymes catalyzing these same reactions have also been found by Gibbs (179) in extracts of pea leaves. Oxidation of fructose diphosphate, fructose-6-phosphate, glucose-6-phosphate, 6-phosphogluconate, and ribose-5-phosphate could be coupled with oxygen uptake when the dialyzed extract was fortified with ATP, DPN, and riboflavin or riboflavin phosphate. Oxidation of these substances was not inhibited by fluoride, azide, and cyanide. Free sugars were not oxidized. Dehydrogenases for glucose-6-phosphate and 6-phosphogluconate, and an enzyme which cleaves ribose-5-phosphate were found in yeasts, animal tissues, and leaves and seedlings of plants by Barkhash & Timofeeva (180).

Cohen (181) has described a pentose isomerase catalyzing the interconversion of D-arabinose and D-ribulose in various strains of *E. coli*. The parent strain, B, does not contain the enzyme, but can synthesize it adaptively if the cells are grown in glucose and arabinose. The isomerase is also present in two mutants of strain B, one of which, selected by growth on D-arabinose, can grow on D-arabinose but not on D-ribose; the other selected by growth on ribose, can grow on either pentose. The parent strain cannot grow on D-arabinose alone because of the absence of a kinase for ribulose, but growth in the mutants is presumably attributable to the presence of a specific ribulokinase, indicated by ability of the arabinose-grown (but not glucose grown) mutants to ferment D-arabinose and D-ribose. Another pentose isomerase, specifically catalyzing the interconversion of D-xylose and D-xylulose, and inactive on other pentoses, was reported by Hochster & Watson (182).

Mitsuhashi & Lampen (183) found the same enzyme activity in *Lactobacillus pentosus* adapted to growth on D-xylose, and they assume that this is the first step in the fermentation of D-xylose in this organism. Lampen (184) has shown that the same adapted organism can convert xylose and xylulose to a mixture of ribose and ribulose 5-phosphates, but no xylose or xylulose phosphates could be detected. These findings would indicate the following steps in xylose fermentation; xylose \rightarrow xylulose \rightarrow ribulose-5-phosphate \rightarrow C₃ + C₂.

Estimation of occurrence of Embden-Meyerhof and shunt pathways.—

Cohen's previous estimation, on the basis of the rate of oxidation of the glucose-1-carbon, that as much as 38 per cent of the glucose dissimilated by *E. coli*, strain B, proceeded via the hexose monophosphate shunt (185) has been verified by subsequent assays of the activity of 6-phosphogluconic acid dehydrogenase in this organism (186). The activity of this enzyme indicated that it could account for a mean of 38 per cent and a maximum of 44 per cent of the total glucose utilization. The studies of Gilvarg (187) with glucose-1-C¹⁴ indicate that the Embden-Meyerhof (E. M.) pathway is predominant in growing cultures of *S. cerevisiae*. This conclusion was based on the observation that the radioactivities of the CO₂, and of the acetate produced, agreed with values to be expected if the E. M. process operated exclusively. In a somewhat more direct study, Blumenthal *et al.* (188) obtained evidence for the appreciable occurrence in yeast of glucose dissimilation mechanisms other than the classical "symmetrical" cleavage of Embden and Meyerhof. Strains of *S. cerevisiae* and *T. utilis* were simultaneously allowed to metabolize separately, glucose-1-C¹⁴ and uniformly C¹⁴-labeled glucose, and respiratory CO₂, alcohol, and acetic acid were isolated and assayed for radioactivity. From the data thus obtained it is possible to calculate the extent of utilization of the glucose 1 carbon for CO₂ and for production of 2-carbon compounds. On the assumption that the E. M. and shunt pathways were the only ones yielding C-2 units, it was calculated that between 30 and 50 per cent of the glucose was dissimilated via the shunt in both fresh and depleted cells, with somewhat higher values for the shunt in *T. utilis* than in *S. cerevisiae*. In similar experiments with *P. aeruginosa*, little if any glucose-1-carbon was used for acetate formation, indicating a negligible occurrence of the E. M. pathway. According to Gibbs & Gastel (189) in the fermentation of glucose-1-C¹⁴ by *Rhizopus oryzae*, the label is present exclusively in methyl groups of ethanol and lactic acid and absent, except for traces, from CO₂. With glucose-3,4-C¹⁴, label was present only in CO₂ and only in carboxyls of lactic acid. Similar findings during oxidative metabolism of labeled glucoses indicates the preponderant occurrence of the E. M. process and very little via the shunt. Similar evidence for exclusive operation of the E. M. pathway in *Clostridium perfringens* was made by Paegle *et al.* (190) who found that fermentation of glucose-3,4-C¹⁴ yielded CO₂ with all of the activity of the labeled glucose and none in ethanol and acetic acid, whereas glucose-1-C¹⁴ or 6-C¹⁴ gave inactive CO₂ when oxidized or fermented by this organism.

In *Penicillium chrysogenum*, de Fiebre & Knight (191) found that about 40 times as much radioactivity from glucose-1-C¹⁴ appeared in respiratory CO₂ as from glucose-2-C¹⁴, indicating predominance of the shunt pathway. Essentially similar conclusions were drawn in experiments with the same organism by Heath & Koffler (192), with the additional information that pyruvate, isolated from this organism metabolizing glucose-1-C¹⁴ in the presence of arsenite, was labeled in the methyl carbon. This indicates the operation of the E. M. process in this organism, and the absence of the

Entner-Doudoroff shunt (*vide infra*). Cochrane *et al.* (193) in similar studies with several species of *Streptomyces* found preferential oxidation of glucose 1 carbon over the 3,4-carbons; the suggestion that the shunt is operating in these organisms was supported by evidence of the presence of key enzymes of this pathway. The same was indicated in *Leuconostoc mesenteroides* by the presence of glucose-6-phosphate dehydrogenase in this organism [DeMoss *et al.* (194)].

Operation of the shunt in animal tissues.—The only available estimations in animal tissues indicate that the shunt does not account for a large proportion of glucose dissimilation in the rat. Bloom *et al.* (195) by measuring the rates of radioactive CO_2 evolution in rats administered glucose-1- C^{14} , uniformly C^{14} -labeled glucose, and C-1, 2, and 3- C^{14} -lactate, were able to calculate that only a negligible proportion of dissimilated glucose was oxidized via the hexose monophosphate shunt. Applying the same procedure to tissue slices, it was found that liver slices utilized glucose preponderantly via the shunt, whereas kidney and diaphragm sections displayed little or no shunt activity. Evidently the metabolic pattern of glucose dissimilation in liver was overshadowed by the predominant occurrence of the E. M. pathway in the musculature. These results were confirmed by a more direct procedure, viz., comparison of CO_2 evolution from glucose-1- and 6- C^{14} (196).

In addition to the E. M. and hexose monophosphate shunt pathways, whose detailed steps have been thoroughly explored, several others have been discovered in certain microorganisms. One of these is a fermentative pathway which leads to the formation of ethanol, the methyl and hydroxymethylene carbons of which are derived respectively from carbons 2 and 3 of glucose; lactic acid, the methyl to carboxyl carbons of which are derived from carbons 4 to 6; and CO_2 , from carbon 1. This process was observed in *L. mesenteroides* by Gunsalus & Gibbs (197) and in *Pseudomonas lindneri* by Gibbs & DeMoss (198). The formation of CO_2 from glucose carbon 1 suggests that a pentose is an intermediate, and this is also indicated by similar types of pentose catabolism in other organisms. Lampen (199) has summarized these in a recent review in which evidence from studies with *Lactobacillus pentosus* indicated that the probable steps were as follows: Ribose-5-P \rightarrow Ribulose-5-P \rightarrow triose-P + "glycolaldehyde" \rightarrow acetaldehyde \rightarrow alcohol or acetate. It is seen that the essential difference between this and the usual hexose monophosphate shunt is the apparent capacity of these organisms for conversion of glycolaldehyde to acetaldehyde. The mechanism of such a transformation is as yet totally obscure. The reviewer would like to make an alternate suggestion that ribulose-5-phosphate may be split between carbons 3 and 4 as well as between carbons 2 and 3. Under these circumstances a triose (in this case dihydroxyacetone) would, by established reactions, give rise to methyl-labeled acetate or ethanol from C-1-labeled pentose. Another possibility would be a conversion of pentose-5-phosphate to the 1-phosphate, followed by splitting between carbons 3 and 4 to yield triose phosphate from pentose carbons 1 to 3. Such a mechanism would explain the findings of Neish

& Simpson (200) (which are otherwise unexplainable by known processes) that C-1-labeled pentose can yield both methyl-labeled acetate and methyl-labeled lactate. These investigators reported that the dissimilation of not only glucose-1-C¹⁴, but also D and L-arabinose-1-C¹⁴ by *Aerobacter aerogenes* yielded acetate, ethanol, 2,3-butanediol, and lactic acid, all of which had in their methyl groups about 40 per cent of the specific activity of the glucose-1-carbon, whereas the carbinol and carboxyl carbons had <1 per cent.

The study of Wood & Harris (201) on formation of acetate from glucose-1-C¹⁴ and 3,4-C¹⁴ in *Clostridium thermoaceticum* is of particular interest because this organism is known to produce three molecules of acetate from each molecule of glucose by a process in which CO₂-fixation is involved. When glucose-1-C¹⁴ was fermented by this organism under conditions in which any assimilated radioactive CO₂ would be highly diluted with ordinary CO₂ the acetate was almost entirely labeled in the methyl carbon. In a similar experiment with 3,4-labeled glucose only slight activity appeared in the acetate. The data indicate that in this organism two acetates arise directly by the E. M. process from carbons 1,2 and 5,6 of glucose, and the third acetate is formed indirectly by reduction of CO₂ derived from carbons 3 and 4.

Another variation of the 6-phosphogluconic shunt has been reported by Entner & Doudoroff (202) to occur in *Pseudomonas saccharophila*. A molecule of glucose (or gluconic acid) is converted to two molecules of pyruvate, one of which isotopic data showed, had its carboxyl group derived from glucose carbon 1. The probable course of events is 6-phosphogluconic → 2-keto-6-phosphogluconic → pyruvic acid + glyceraldehyde-3-phosphate → pyruvic acid. Unpublished studies with glucose-1-C¹⁴ by Blumenthal & Lewis in the reviewer's laboratory extended this process to *P. aeruginosa*.

The formation of keto acids from glucose is emphasized in several other studies. Wood & Schwerdt (203) reported a system in sonically-destroyed cells of *Pseudomonas fluorescens* which oxidizes glucose and gluconic acid to 2-ketogluconate. The system apparently does not involve any of the known phosphorylated intermediates of glucose catabolism. Sokatch & Gunsalus (204) have shown that *S. faecalis* 10C1 can be adapted to pathways other than the Embden-Meyerhof, to ferment gluconate, 2-ketogluconate, and 5-ketogluconate. Fermentation of gluconate yielded 1.5 moles of lactate, 0.5 moles of CO₂, and an unidentified acid. Preferential utilization of gluconate-1-carbon for CO₂ formation suggested the occurrence of a pathway similar to the oxidative shunt. Simultaneous adaptation of gluconate-grown cells to 2-ketogluconate indicates that this keto acid is on the direct pathway of gluconate metabolism. Another keto acid product of the metabolism of gluconate in *Acetobacter melanogenum* has been tentatively identified by Katznelson *et al.* (205) as 2,5-diketogluconate.

The possibility has often been under consideration that sugars may be broken down by successive, stepwise decarboxylations of aldonic acid phosphates. Glock (175) has provided data which indicate that the occurrence of

such a process beyond the pentose stage is unlikely, at least in liver. Oxidation of ribose-5-phosphate required two fractions of horse liver, one of which, B, was most active in splitting pentose phosphate, the other had high glucose-6-phosphate dehydrogenase activity. However, these fractions, separately or combined, did not oxidize 5-phosphoribonic acid. The main product of fraction B action on ribose-5-phosphate was fructose monophosphate, which was gradually converted to glucose-6-phosphate. The inertness of fructose-1,6-diphosphate in this system precluded its participation in this conversion and excluded the possibility that hexose formation may have been a result of the action of aldolase on triose phosphate. Since the high yield of hexose phosphates (75 per cent of pentose carbon utilized) is incompatible with their formation solely from triose phosphate, the participation of the 2-carbon moiety was also indicated. The possibility was considered that the dehydrogenase fraction, B, might act upon the 2-carbon moiety, but glyceraldehyde could not be detected, nor was it oxidized by fraction B. When glyceraldehyde was incubated with triose phosphate or ribose-5-phosphate a ketopentose-1-phosphate rapidly accumulated, but no evidence was obtained for the accumulation of such a substance during breakdown of ribose-5-phosphate. These results are readily explainable on the basis of the transaldolase and transketolase reactions already discussed.

In an exploration of intermediates of carbohydrate oxidation in *Aerobacter cloacae* De Ley (206) found that whole cells of this organism oxidized D-glucose, D-fructose, dihydroxyacetone, L-ascorbate, and glyceric acid without a lag period, indicating the constitutive nature of enzymes responsible for their oxidation. A larger group of substances was oxidized after a lag period, indicating adaptation: viz., D-gluconate, 2-keto-D-gluconate, glucuronate, saccharate, D-galactose, D-galactonate, galacturonate, D-mannitol, D-mannose, D-sorbitol, 2,3-diketo-L-gulonate, 2,3-diketo-D-mannonate, D-xylose, D-lyxose, L-arabinose, D-ribose, glycerol, and D-glyceraldehyde. Another group was not oxidized, consisting of 5-ketogluconate, kojate, mucate, D-mannonate, mannuronate, L-sorbose, D-gulonate, D-araboascorbate, oxalate, D-xylonate, D-arabinose, D-arabonate, L-arabonate, D-ribonate, L-xylose, α -glucoheptonate, i-erythritol, glycollate, and ethylene glycol. This author has also shown that adaptation to the sugars involves the synthesis of kinases which phosphorylate gluconic, galactonic, and 2-ketogluconic acids, D-ribose and D-xylose (207, 208).

METABOLISM OF SUGARS AND THEIR INTERMEDIATES IN ANIMAL TISSUES

Hormonal effects on sugar utilization by tissues.—A number of studies have been recorded in which the rat diaphragm has been utilized to study the effects of hormones or other factors on various aspects of glucose metabolism. Additional evidence of an effect of insulin on the hexokinase reaction has been provided by Mackler & Guest (209). Although the utilization of fructose by rat diaphragm was accelerated by insulin, just as is the utiliza-

tion of glucose, insulin had no effect on fructose utilization when this sugar was accompanied by glucose. The authors explain these results on the basis that in the absence of glucose, fructose is phosphorylated by hexokinase, which is responsive to insulin. In the presence of glucose, fructose utilization via hexokinase is inhibited, but proceeds via the specific fructokinase, which is not inhibited by glucose and which is unresponsive to insulin. A puzzling feature of these results, however, is the observation that fructose utilization in the presence of glucose was just about as great as in its absence. If glucose were inhibiting an important, insulin-responsive pathway of fructose utilization, it would be expected to lower the disappearance of fructose.

In previous studies Stadie and co-workers showed that insulin could be bound by isolated rat diaphragm in a form which resisted subsequent washing; such bound insulin exerted a marked acceleration of glycogen synthesis from exogenous glucose. In an extension of this technique, Stadie *et al.* (210) made some significant observations which illustrate the interplay of hormones on glycogen deposition. The insulin effect was considerably enhanced over the normal in the hypophysectomized but was unchanged in the adrenalectomized rat. Prior treatment of normal rats with growth hormone or cortisone decreased the action of insulin somewhat. Similar treatment of the hypophysectomized or adrenalectomized rat either had no effect or increased that of insulin, but administration of both hormones together resulted in a marked lowering of the insulin effect. Although the mechanism of these effects remains unexplained, they provide further examples of antagonism between insulin and the other hormones.

Further evidence of the inhibiting effects of adrenal hormones on glucose metabolism has been provided by Grossman *et al.* (211). Using C^{14} -labeled glucose with isolated rat diaphragms *in vitro*, it was found that cortisone and hydrocortisone lowered glucose uptake, glycogen synthesis, and $C^{14}O_2$ production. Corticosterone depressed only $C^{14}O_2$ production, whereas desoxycorticosterone inhibited only glycogen synthesis. These findings suggest that the various adrenal steroids have different sites of action in carbohydrate metabolism.

Antagonistic effects between these hormones are also suggested by the findings of Kovach *et al.* (212) concerning glucose uptake by rat diaphragm in shock. Diaphragms from normal shocked animals showed a decreased glucose uptake which was restored to normal by addition of insulin to the medium. No decrease occurred in shocked hypophysectomized or adrenalectomized rats. In a study of the effect of glucose concentration on its uptake by rat diaphragm *in vitro* Torres (213) found that the ionic composition of the medium greatly influenced glucose uptake.

Beloff-Chain *et al.* (214) have made some surprising observations concerning the action of insulin on the metabolism of glucose phosphates in rat diaphragm. In contrast with its action on glucose, insulin inhibited uptake of glucose-1-phosphate and had no effect on glycogen synthesis therefrom. Oxygen consumption of diaphragm was not increased by glucose but

was considerably increased by glucose-1-phosphate. However, glucose-6-phosphate affected neither oxygen consumption nor glycogen synthesis, but apparently entered the cells, since it was rapidly converted to an equilibrium mixture with fructose-6-phosphate.

Insulin and cellular permeability.—It is becoming increasingly apparent that at least one function of insulin is in regulating cellular permeability to glucose. Perhaps the most convincing evidence for this action of insulin is its ability to increase the volume of distribution of nonutilizable sugars such as galactose in the whole animal. The original finding by Levine *et al.* (215) that administration of excess insulin almost doubled the volume of distribution of galactose in the intact dog have now been extended to other sugars. Goldstein *et al.* (216) found that L-arabinose and D-xylose responded to insulin as did galactose, whereas D-arabinose and L-rhamnose were not responsive to insulin. These authors pointed out that the sugars which exhibit this insulin effect have the glucose configuration of carbons 1 to 3. The same authors (217) found that muscular work also simulates the action of insulin in increasing the volume of distribution of these sugars, an effect which is not attributable to elicitation of insulin secretion, since it occurs in the depancreatized dog. Apparently muscular work promotes a factor, other than insulin, which enhances cellular permeability to sugars, or it results in the removal of substances inhibitory to insulin. The action of insulin in increasing the volume of distribution of galactose was confirmed by Wick & Drury (218) in experiments with C^{14} -labeled galactose in eviscerated rabbits. An exaggerated effect of insulin was obtained, amounting to a volume of distribution of 157 per cent. This anomalous result indicated that a large proportion of the galactose, when it entered the cell in response to insulin, was changed chemically; in agreement with this probability there was observed a small but definite oxidation of galactose, indicated by appearance of radioactivity in the respiratory CO_2 . That the primary effect of insulin in increasing utilization of glucose by rat diaphragm is to increase cellular permeability rather than specific intracellular metabolic processes is also indicated in a study by Haft *et al.* (219) who found that whereas insulin increased the uptake of glucose, fructose, D-galactose, and D-arabinose by rat diaphragm *in vitro*, only the former two sugars were utilized to an extent greater than could be accounted for by diffusion into the cells. Ross (220) found that addition of insulin to the incubation medium caused a 3-fold increased uptake of glucose by isolated whole rabbit lenses. Under similar conditions only a slight acceleration was observed in lens homogenates; again this emphasizes the role of insulin in increasing cellular permeability to glucose.

The long-standing question of whether glucose absorption is an energy-requiring chemical process seems now to be answered in the affirmative at least for the intestine, as the result of a recent study by Darlington & Quastel (221).

These investigators devised a system whereby the transfer of sugars across the intestinal wall could be studied in relation to respiratory activity. The

dependence of glucose absorption on respiratory activity was strikingly demonstrated. The absorption rates in relative values were glucose, 100; galactose, 100; fructose, 50; and sorbose, 7. The absorption of the sorbose was assumed to be attributable to diffusion only. With nitrogen as the gas phase, absorption of glucose was cut to about one-fifth of its level in oxygen; similar or greater inhibitions were observed in the presence of 0.01 *M* cyanide, azide, chloretone, and 0.001 *M* 2,4-dinitrophenol and 0.0025 *M* quinine; 0.01 *M* sodium fluoroacetate and malonate inhibited to a lesser extent. D-Glucosamine and fructose diphosphate were not appreciably selectively absorbed, but glucose-1-phosphate was absorbed rapidly, and its absorption was also inhibited by anaerobiosis. These data clearly establish the process of absorption of certain sugars across the intestinal wall as being respiration-dependent, and the inhibitory effects of dinitrophenol implicate phosphorylations in the mechanism. Essentially the same conclusions were drawn from similar experiments by Hestrin-Lerner & Shapiro (222). These investigators found, using C^{14} -labeled glucose, that a large proportion of the absorbed sugar was in a nonfermentable form, which was not further identified. If these findings are to be regarded as typical of glucose absorption generally, it is possible that the effects of insulin on glucose phosphorylation and its absorption by cells are exerted on the same underlying process. Evidence of the possibility that hexokinase activity may be involved in the intestinal absorption of glucose is available. Long (223) found that the hexokinase activity of intestinal mucosal homogenates of the rat could be increased by prior feeding of high carbohydrate, and decreased by prior feeding of low carbohydrate diets. The effect was specific for hexokinase and for the mucosal layer; it was not observed for alkaline phosphatase activity nor for the musculature of the intestine.

In another study of intestinal absorption of glucose by intact guinea pigs, Lourau (224) found that if the blood sugar of insulinized animals was raised by glucose administration to the level of normal animals, glucose absorption was lowered to values given by normal animals having the same blood glucose level. The conclusion was drawn that insulin exerts only a secondary effect on glucose absorption by its action on the blood sugar.

Respiration and glycolysis of glucose in animal tissues.—Terner (225) found marked differences between mammary gland slices of rat and rabbit in their ability to glycolyze glucose anaerobically. Whereas the former was active in the presence of nicotinamide and pyruvate, the latter was much less active, even when fortified with ATP and DPN⁺ (it is likely, however, that these nucleotides were not being absorbed). This difference was attributed to the prevalence of different pyridine nucleotidases in the two tissues: the nicotinamide-sensitive enzyme in rat tissues, and the nicotinamide-insensitive DPN⁺ pyrophosphatase in rabbit tissue. In addition to DPN⁺ breakdown, the overall process was limited by hexokinase activity, inasmuch as fructose-1,6-diphosphate was glycolyzed more rapidly than glucose; the addition of yeast hexokinase and glucose greatly increased glycolysis over

that observed with glucose alone. With the aid of C^{14} -labeling, Wenner *et al.* (226) established conditions for maximal oxidation of glucose and fructose in whole homogenates of rat and mouse tissues, the oxidation rates being measured, independently of manometric data, by the incorporation of C^{14} in respiratory CO_2 . All tissue studied, comprising heart, kidney, brain, and liver, required exogenous DPN⁺, but added ATP had little or no stimulating effect except in brain. The system required Mg, K, and phosphate ions and was optimal with isotonicity of incubation and homogenization media. The most striking observation was the lack of influence of exogenous ATP on glucose oxidation in heart and kidney, which the authors believe is a result of an efficient, self-contained glucose phosphorylation mechanism within the mitochondria. This conclusion is supported by other observations. Crane & Sols (227) reported that a large fraction of the hexokinase present in various rat tissues is associated with the cell fraction sedimentable at relatively low speed, and Saltman (228) has also found soluble and insoluble forms of hexokinase in a variety of tissues of higher plants. Mouse brain mitochondria carried out the oxidation of glucose without exogenous supplementation of enzymes, in the presence of DPN⁺, ATP, Mg ions, and nicotinamide, according to Hesselbach & DuBuy (229), again demonstrating the presence of particle-bound hexokinase in this tissue.

Further evidence for abnormally high gluconeogenesis in diabetes has been provided by Renold *et al.* (230) at the tissue slice level. Liver slices of rats rendered diabetic by alloxan injection or pancreatectomy produced more glucose from added pyruvate, from tissue glycogen, and from other, nonidentified endogenous sources, then comparably fed normal rats. This effect was diminished when the diabetic condition was complicated by superimposed adrenalectomy or hypophysectomy.

Pyruvate metabolism.—In a comprehensive study of the metabolic fates of glycerol and pyruvate in rat liver slices, using C^{14} -labeling, Teng *et al.* (231) found that qualitatively the metabolism of both substrates followed a similar course; however, considerably more glycerol than pyruvate was converted to lactate and CO_2 . Under the conditions of the experiments most of the lactate was derived by direct reduction of pyruvate. Using 2-labeled pyruvate only very little radioactivity appeared in lactate carbon 1, and there was virtually no randomization of activity between carbons 2 and 3. These results indicate that there is little resynthesis of pyruvate from 4-C acids; hence little occurrence of a dicarboxylic acid cycle in liver cells.

Miller & Olson (232) made a detailed study of the oxidation of pyruvate and DL-lactate in slices of duck heart, using C^{14} -labeling. The authors found that utilization of these metabolites paralleled their concentration in the medium, in confirmation of previous studies in whole animals or heart-lung preparations. Increasing concentration of substrate resulted in its increased disappearance, presumably by transformation to carbohydrate or glucose, since there was no increase in conversion to CO_2 . However, it was established, by comparison of oxygen consumption with the extent of conversion

of carbons 1 and 2 of pyruvate to respiratory CO_2 , that a large fraction of utilized pyruvate was oxidized to CO_2 . As the substrate concentration was increased from 5 to 40 mM per liter, the contribution of pyruvate oxidation to the total oxygen consumption increased from 45 to 93 per cent. This finding well illustrated the fallacy of calculating the degree of oxidation of substrates by increases in oxygen consumption over endogenous values. An interesting comparison of the metabolism of D(-) and L(+) lactates in various tissues was made by Brin *et al.* (233). Though the physiological L isomer was oxidized much more rapidly than the D isomer in all tissues studied (heart, liver, and brain slices of the rat and duck), there was a large, non-oxidative utilization of D(-) lactate, presumably for glycogen formation, which reached a magnitude approaching that of the L enantiomorph, particularly in heart slices. The authors point out that these observations cannot be accounted for solely by the action of a racemase on the D isomer. In a study of pyruvate oxidation in mitochondria of sheep and ox kidney, Bartley (234) found that the requirement of fumarate for complete oxidation of the keto acid could be replaced by bicarbonate, indicating the presence of a carboxylating system in these tissues leading to synthesis of oxalacetate. In the absence of added bicarbonate or fumarate, acetate accumulated.

Acetate oxidation.—Wick & Drury (235) found that musculature of the eviscerated rabbit has a very high capacity for acetate oxidation which can be evoked by administration of large amounts of acetate. They estimate that at least half of the energy needs of the tissues can be supplied by this acid. The simultaneous administration of insulin and glucose does not lower the rate of acetate oxidation in this preparation (236).

METABOLIC TRANSFORMATIONS INVOLVING GLUCOSE

Interconversion of sugars—fructose to glucose.—It is now apparent that the conversion of fructose to glucose, formerly thought to proceed in liver via the steps: fructose→fructose-1-phosphate→fructose-6-phosphate→glucose-6-phosphate→glucose, may not be so direct. Leuthardt *et al.* (237) summarized and extended their studies of the metabolism of fructose-1-phosphate, the ester formed directly by fructokinase action in liver, and described an aldolase in rat liver which splits this ester into one molecule each of dihydroxyacetone phosphate and free glyceraldehyde. The equilibrium of the reaction is far toward the hexose phosphate; in dialyzed preparations, therefore, no reaction occurs unless DPN is added. The coenzyme activates the reaction by promoting the dismutation of the triose products, glyceraldehyde to glyceric acid and dihydroxyacetone phosphate to α -glycerophosphoric acid. Using a purified preparation of the aldolase, together with DPNH and glycerophosphate dehydrogenase, the aldolase reaction can be measured optically by oxidation of DPNH. These authors believe that the conversion of fructose-1-phosphate to glucose-6-phosphate, which was found by Cori *et al.* (238) to occur in a fraction of rat liver, and which they attributed to the successive actions of a phosphofructomutase and phosphohexose iso-

merase, is in reality a consequence of the following successive actions: triose phosphate formation via "1-phosphofructaldolase," condensation of the triose phosphate by means of the classical aldolase to hexose diphosphate, and dephosphorylation of the latter to fructose-6-phosphate. In support of this hypothesis they offer the following observations: (a) a specific phosphatase was obtained, free from aldolase, which rapidly converted fructose-1,6-diphosphate to fructose-6-phosphate; (b) by combining this purified phosphatase with 1-phosphofructaldolase, muscle aldolase, and triose phosphate isomerase, fructose-1-phosphate was converted to the 6-ester; (c) no evidence was found for the direct conversion of the 6-ester to the 1-ester, a reaction which is favored thermodynamically and should, therefore, have occurred in the presence of a "phosphofructomutase."

Also explainable in terms of reactions catalyzed by the classical aldolase and the 1-phosphofructaldolase is the reversible conversion of fructose-1,6-diphosphate to fructose-1-phosphate in the presence of glyceraldehyde, reported by Cardini (239) to be catalyzed by an aldolase preparation from jack beans.

Glucose to glucosamine.—According to Becker & Day (240) glucosamine is formed in the rat by direct transformation from glucose without cleavage, a probable intermediate being glucosone. When glucose-1- C^{14} was fed, radioactivity was present in carbon 1 of serum glucosamine; when glucosone-1- C^{14} was fed, a considerably greater percentage of administered radioactivity appeared, likewise in carbon 1 of serum glucosamine. Apparently glucosone can undergo further metabolism in the rat, since it is converted to CO_2 , though at about one-third the rate of glucose, and is also converted to liver glycogen. Rieder (241) tested the possibility that glucosamine might arise from triose and serine by an aldolase type of reaction; however, the data were again in better accord with a direct transformation from glucose. Both glucose-1- C^{14} and $N^{15}H_3$ were rapidly incorporated into glucosamine of ovomucoid of eggs laid by hens given these substances, whereas glycine- $N^{15}C^{14}$, a precursor of serine, was a relatively poor source of labeled glucosamine. Roseman *et al.* (242) also found that glucosamine, recovered from hyaluronic acid synthesized by group A *Streptococci*, in the presence of glucose-1- C^{14} , was labeled entirely in carbon 1. These investigators also found that the N-acetyl moiety had essentially the same activity as the initial glucose, with all of its activity in the methyl carbon, in accord with its formation via the Embden-Meyerhof pathway of glucose dissimilation.

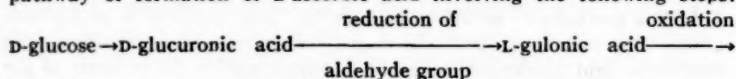
The presence of uridine diphosphate acetylglucosamine (UDPG) in yeast led Cabib *et al.* (243) to consider the possibility that this pyrimidine nucleotide plays a part in the transformation of glucose to glucosamine. Enzymes were found in *Neurospora crassa* which converted hexose-6-phosphate to glucosamine, acetylated the latter, and interconverted acetylglucosamine-1- and 6-phosphates (244), but no evidence was found for the participation of UDPAG in these reactions.

Glucuronic acid.—That glucuronic acid is also formed by direct trans-

formation of the glucose carbon chain has been confirmed in several studies. According to Roseman *et al.* (245) the hyaluronic acid synthesized by group A *Streptococci* from C-6-labeled glucose contained glucuronic acid labeled only in the carboxyl carbon. Douglas & King (246) fed borneol together with glucose 1- or 6-C¹⁴ to guinea pigs and isolated the bornylglucuronide from the urine. With each labeled substrate, 67 per cent of the radioactivity of the glucuronide was present in the corresponding 1 or 6 position. As the authors point out, this consistent retention of activity in a single position, constitutes strong evidence for a direct transformation without cleavage of the hexose carbon skeleton. Apparently glucuronic acid can be extensively metabolized in the guinea pig and rat. In contrast with findings of Packham & Butler (247), Douglas & King (248) observed a rapid conversion of the labeled glucurone to CO₂, with a higher recovery from 6-labeled than from uniformly labeled glucose, indicating a preferential oxidation of carbon 6.

Somewhat surprising was the finding that preformed glucuronic acid, when fed to rats or guinea pigs, is not conjugated with borneol. Little or no radioactivity from glucurone-6-C¹⁴ appeared in the bornylglucuronide. Though the uniformly labeled glucurone did yield labeled conjugate, the C¹⁴ was unsymmetrically distributed; about twice as much activity was found in carbons 1 to 3 than in carbons 4 to 6, indicating that the transformation was indirect. Evidently glucuronic acid yields a 3-carbon fragment having a preference for the first three carbons of the glucuronide chain. The authors suggest that glucuronic acid may yield a molecule of dihydroxyacetone phosphate which condenses with 3-phosphoglyceraldehyde from the body pool more rapidly than it is isomerized by triose phosphate isomerase, thus yielding fructose diphosphate labeled preponderantly in the first three carbons. Supporting this postulation is the formation of similarly unsymmetrically labeled glycogen from the uniformly labeled glucurone.

Glucose to L-ascorbic acid.—Isherwood *et al.* (249) suggested a possible pathway of formation of L-ascorbic acid involving the following steps:



L-ascorbic acid. In support of this reasonable formulation they found that D-glucuronic, L-gulonic, and L-galactonic acids were converted to L-ascorbic acid when administered to cress seedlings or to rats, whereas all of the other D- and L- hexonic acids were inactive. This postulation is in accord with the results of Horowitz *et al.* (250, 251) who found that in the chloretone-treated rat, administration of glucose-1-C¹⁴ yielded L-ascorbic acid labeled mainly in the 6 position, whereas glucose-6-C¹⁴ yielded L-ascorbic acid-1-C¹⁴. The similarity in radioactivity yields from the two precursors and the similar distribution of activity in the remaining carbons provide strong evidence that conversion occurs in the main without cleavage of the carbon chain and with maintenance of the steric configuration around carbons 4 and 5 of ascorbic acid (carbons 2 and 3 of glucose) as would be re-

quired by the suggestion of Isherwood *et al.* That one of the intermediates of L-ascorbic acid synthesis is 2,3-diketo-L-gulonic acid has been demonstrated by Sasamoto *et al.* (252) whose results are available only in abstract form. This acid was found in rat and rabbit liver and lens, but not in tissues of the guinea pig and was shown to be converted to ascorbic acid in extracts of rabbit and rat liver, but not by guinea pig liver.

Glucose to gluconic acid.—Despite the presence of glucose oxidase in animal tissues, the product of its action on glucose, viz., gluconic acid, is apparently not produced in appreciable quantities in the intact animal. According to Stetten & Topper (253), C^{14} -labeled gluconate administered to rats is excreted in the urine without dilution of its specific activity. In most experiments the amounts excreted ranged between 60 and 85 per cent of the dose given. The remainder was metabolized, being recovered as CO_2 and as liver glycogen. In normal rats the recovery of gluconate-1-carbon as CO_2 exceeded that from the remainder of the molecule; in diabetic rats, however, more radioactive CO_2 appeared from uniformly-labeled than from C-1-labeled gluconate. An extremely low recovery of activity from gluconate-1- C^{14} in liver glycogen from normal rats and in urinary glucose from alloxan-diabetic rats suggested that the direct reduction to glucose is not a quantitatively significant reaction; indeed most of the gluconate-1-carbon appearing in glucose could be attributed to CO_2 -fixation at the 3-carbon level. However, gluconate can be broken down to smaller fragments which can be resynthesized to glucose, inasmuch as relatively large amounts of carbon from uniformly-labeled gluconate were recovered in liver glycogen or urinary glucose of diabetic rats.

The apparent physiological inactivity of the liver glucose dehydrogenase has been found by Brink (254) to be a result of its low affinity for the substrate; hardly any oxidation occurs below 0.01 *M* glucose concentration, and it is powerfully inhibited by glucose-6-phosphate at levels of 10^{-6} *M*.

Glucose to kojic acid.—Arnstein & Bentley (255) applied the C^{14} -tracer technique to a study of kojic acid synthesis in species of *Aspergillus*. Though their results make it appear quite likely that kojic acid is produced from glucose by a direct transformation, they do not entirely rule out the possibility that the hexose chain may be broken and recombined in the process.

Glucose to phenylalanine.—Gilvarg & Bloch (256) having grown yeast on glucose-1- C^{14} and acetate-1- C^{13} as the principal sources of carbon, found that whereas acetate carbon was not significantly incorporated into phenylalanine and tyrosine isolated from the cell proteins, glucose carbon was incorporated highly and with a specificity indicating a rather direct conversion. Nearly all of the radioactivity was present in the β -carbon of the side chain and in positions 2 or 6 (or both) of the ring (because of the symmetry of the amino acids, these positions cannot be distinguished). The activity distribution in the side-chain clearly indicates its origin from a 3-carbon compound, but the activity distribution in the ring excludes the involvement of C 2 units, acetate, intermediates of the citric acid cycle, or isotopically equi-

brated 3-carbon compounds. The possibility of a direct cyclization of the hexose chain was dismissed because the ring carbons had only 60 to 70 per cent of the activity of the initial glucose, or that of the glucose and mannose of the yeast polysaccharides. In considering various possible mechanisms the author suggest that a 7-carbon sugar, perhaps sedoheptulose, arising as discussed previously from a tetrose and triose, may be the precursor of shikimic acid, the 7-carbon compound produced by tyrosine-requiring mutants of *E. coli*. Results of a study of the biosynthesis of shikimic acid in this mutant by Shigeura *et al.* (257) also indicate that the intact hexose chain does not undergo cyclization to form shikimic acid. When derived from glucose-1- C^{14} grown cells, the radioactivity was predominantly in C 2 of shikimic acid with one-half the activity of the glucose-1-carbon in this position; when grown on C-6-labeled glucose the activity was mainly in C-6 with only 15 per cent dilution. With glucose-3,4- C^{14} , activity was divided among carbons 4 and the carboxyl (one-third each) and carbons 3 and 5 (one-sixth each). Thus two or more fragments of the hexose chain must combine in the biosynthesis, and in a manner such that carbon 6 is better utilized than carbon 1. Though both of these studies are consistent with the utilization of the shikimic acid ring as the precursor of the phenylalanine ring, the carboxyl carbon of shikimic acid is evidently not the precursor of the β -carbon of the aromatic amino acids, since C 1 of glucose did not appear in the shikimic acid carboxyl, as it did in the phenylalanine β -carbon. Ehrensverd & Reio (258) analyzed their extensive data on the distribution of isotopic carbon in tyrosines isolated from *Rhodospirillum rubrum* grown under anaerobic conditions on variously labeled acetates and $C^{14}O_2$. Although the data did not allow any decisive conclusions (principally because of the symmetry of the benzene ring which makes it uncertain whether activity in the carbon pairs, 2,6, and 3,5 are in either or both carbons of each pair) various alternatives were considered. One of these alternatives was in fair accord with a mechanism involving the formation of hexose from acetate carbon and CO_2 by known pathways, followed by splitting of the hexose and recondensation of C 3 and C 4 or C 5 and C 2 fragments to yield a 7-carbon compound making up the ring and the β -carbon atom of the aromatic amino acids.

REVIEWS OF INTERMEDIARY METABOLISM

In addition to those already cited, excellent reviews of many aspects of carbohydrate metabolism in microorganisms are found in a Symposium on Some Aspects of Microbial Metabolism (259) including the following: Van Niel, Comparative Biochemistry of Microorganisms; Umbreit, Respiratory Cycles; Stadtman, Synthesis of Some Coenzyme A Compounds; Gunsalus, The Chemistry and Function of the Pyruvate Oxidation Factor; Horecker, Metabolism of Pentose Phosphate; Wood & Schwerdt, Alternate Pathways of Hexose Oxidation in *Pseudomonas Fluorescens*; Lampen, Pentose and Desoxypentose Metabolism in Bacteria; DeMoss, Routes of Ethanol Formation in Bacteria; Leaver & Wood, Propionic Acid Fermentation. A

comparable survey of carbohydrate metabolism in animal tissues (260) contains the following topics: Soskin, The Production, Disposal and Regulation of the Blood Glucose; Wick & Drury, The Effect of Competitors on Glucose Oxidation; Strisower & Searle, Turnover and Oxidation of Glucose in Normal and Diabetic Animals; Chernick, The Effect of Insulin on Hepatic Utilization of Glucose; Levine & Goldstein, The Effect of Insulin on the Rate of Transfer of Sugars Across the Cell Barriers; D. Stetten, Jr., Glucose Synthesis and Oxidation in the Alloxan-Diabetic Rat; Handler, Protein as a Metabolic Fuel; M. R. Stetten, A Study of the Metabolism of Gluconic Acid; Dickens, The Significance of the Direct Pathway of Glucose Oxidation; Brady & Gurin, The Biosynthesis of Fatty Acids in Cell-Free Systems; Sakami & Rudney, The Metabolism of Acetone and Acetoacetate in the Mammalian Organism; Korkes, A Potential Pathway from C_2 to C_3 and The Question of Gluconeogenesis from Fatty Acids; Weinhouse, Factors Involved in the Formation and Utilization of Ketone Bodies. Other reviews have appeared by, Lipmann, The Chemistry and Function of Coenzyme A (261); Barron, The Oxidative Pathways of Carbohydrate Metabolism (262); DeLey, L'oxydation directe du glucose par les microorganismes (263), and Ruzicka, The Isoprene Rule and The Biogenesis of Terpenes (264).

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CHEMISTRY OF THE PROTEINS, PEPTIDES, AND AMINO ACIDS^{1,2}

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In view of the amount of material reasonably assignable to this chapter and with the protection of the comprehensive coverage of last year's review by Fromageot & Justisz (1), the authors have chosen to delimit drastically the subject matter of this review in the interests of coherence. Thus while all enzymes are proteins, enzymes will here be considered only in regard to non-enzymic properties and the reader is referred to other chapters in this volume dealing with enzyme function. Similarly, muscle proteins have been relinquished to the chapter on muscle, and iodo-proteins and iodinated amino acids to the chapter on iodine metabolism. Moreover, several topics of widespread interest have nevertheless been avoided because of their very magnitude, even without the hope that they will be covered elsewhere in this volume. Thus optical and x-ray studies have been omitted, and the reader is referred to the texts and symposia cited in the following paragraphs.

During the past year several excellent textbooks, reviews, and symposia on various aspects of protein chemistry have appeared. Most outstanding of these is the comprehensive treatise *The Proteins* (2) in two volumes, of which Volume I, parts A and B, have already appeared. Since this may serve as a standard reference for some years, an outline of its contents would appear desirable. Taylor discusses the isolation of proteins, emphasizing the maintenance of biological activity (i.e., prevention of denaturation) and methods of following degrees of purification achieved. While attempting to cover all methods of purification, he has perhaps slighted conventional methods based on protein solubility in favor of the newer counter-current procedures and electrophoresis. Desnuelle gives a potpourri of the chemistry of the amino acids which is particularly pertinent to the chemistry of proteins. He has discussed protein hydrolysis and the isolation and determination of structure of peptides. Tristram has critically evaluated the accuracy of amino acid analysis and has compiled tables of amino acid composition of approximately 50 proteins. These data are an enlargement of those previously assembled by him (3). He goes on to discuss patterns in the distribution of amino acids in various proteins after the manner of Bailey (4). Low has given a comprehensive review of amino acid and protein structure as elucidated from x-ray diffrac-

¹ The literature survey for this review was concluded in December, 1953.

² The following abbreviations are used: DNFB for 2,4-dinitrofluorobenzene; DNP for dinitrophenyl.

tion studies and other crystallographic properties. She has done an excellent job of making the methods and results of these studies comprehensible to the noncrystallographer. Doty & Geiduschek discuss the contribution to structure from studies of the optical rotation, optical absorption, and diffraction of proteins. Alberty, after a brief discussion of hydrogen ion equilibria as revealed by potentiometric titrations, covers in some detail the electrophoretic mobility of proteins, giving the theoretical background necessary for the understanding and use of electrophoresis for protein purification and characterization.

Edsall opens Volume I, part B, with an excellent review of the methods of measurement, data, and concepts relating to the size, shape, hydration, and dielectric increment of proteins. This chapter may best be described as a condensation of the protein section of Cohn & Edsall's text (5) brought up to date. Klotz discusses the interaction of proteins with ions, illustrating the statistical and thermodynamic calculations involved. Putnam gives a lengthy description of the phenomenon of protein denaturation. In the following chapter, Putnam discusses chemical modification of proteins, giving in more detail the material covered in earlier reviews by Herriott (6) and Olcott & Fraenkel-Conrat (7). Additional reagents discussed include dinitrofluorobenzene (DNFB)², diisopropyl fluorophosphate, carboxypeptidase, and the enzyme from *Bacillus subtilis* which converts ovalbumin to plakalbumin. In the last chapter, Porter sums up Volume I, the general chemistry of proteins, with a quest for the explanation of biological activity in terms of structure. His search is as frustrated as that of others before him.

The editors are to be complimented on the choice of subject matter for this treatise and in the lucidity of the presentations. It is to be hoped that the second volume (due early in 1954) dealing with "specific proteins which either by circumstance of occurrence, role, chemical similarity, or mechanical functions, are best considered together" will be of similar excellence.

A book entitled *The Mechanism of Enzyme Action* (8), a collection of papers from a symposium held at the McCollum-Pratt Institute of the Johns Hopkins University, in June, 1953, is considered outside the range of this review, excepting for contributions by Kirkwood & Kauzmann. Kirkwood further expands his concept of a fluctuating charge on the protein molecule arising from proton migrations among a number of sites of similar basicity, which are not all saturated. Besides the effect on the dielectric increment already predicted (9), he now feels this effect may contribute largely to protein-protein interaction via induced polarization (by proton displacement) in suitably complementary structures. Kauzmann gives a clear and well coordinated picture of protein denaturation, discussing the mechanism and emphasizing the roles of hydrogen-bonded peptide chains and disulfide linkages in the native structure. The experimental details on which these concepts are based have recently been published (9a to 9e).

In one of the Lane Medical Lectures of October, 1951 (10), Linderström-Lang discussed the denaturation which accompanies the early stages of pro-

teolysis. He cites the recent work of Korsgaard Christensen on the denaturation of β -lactoglobulin with trypsin (11), which still does not answer the question whether such denaturation can take place without splitting any peptide bonds. In another lecture he reviews the enzymatic production of plakalbumin from ovalbumin, the nature of the peptide split-products, and their possible linkage to the protein. In the final lecture he gives a beautiful general survey of postulated mechanisms for protein biosynthesis. (Other lectures in this volume pertain to his micromethods of studying metabolism and the results so obtained.) This volume is particularly recommended to the general reader because of its clarity and breadth of view.

A symposium on blood proteins held in Cohn's laboratory during 1951 to 1952 has now appeared in print (12). Largely limited to human blood, it contains chapters on: new methods of separation of plasma components (use of metallic ions, ion-exchange resins, adsorbents, etc.) by Cohn *et al.*; some aspects of the clotting process, by Alexander, Ferguson & Edsall; antibodies (their function, properties and formation), by Janeway, Enders, Oncley & Ehrlich; the properties of plasma lipoproteins and their relation to other lipoproteins, by Oncley, Gurd, Macheboeuf & Folch-Pi.

A symposium on immunochemistry held by the Biochemical Society in November, 1952, has been published (13), as well as symposia by the Royal Society in the same year on "the structure of proteins" (14) and a general discussion of protein chemistry by the Faraday Society (15). The Royal Society meeting centered its attention on the helices of Pauling and Corey and was largely concerned with the interpretation of x-ray data. *The Discussions of the Faraday Society* contains much interesting reading and a wealth of isolated observations which it would be impossible to summarize here. The section on conjugated proteins included a stimulating review by Stacey.

Advances in Protein Chemistry, Volume 7 (16), contains a clear exposition by Sanger of the organic chemist's approach to the "arrangement of amino acids in proteins." While it was written too soon to include his determination of the order in the A chain of insulin (17), the various techniques leading up to this magnificent achievement are described. In this volume there is also an excellent chapter on "the structure of the collagen fibril" by Bear, who has gone to considerable effort to correlate chemical data with optical (electron-microscopy and x-ray diffraction). There is also a detailed discussion by Beaver & Holiday of the "ultra-violet absorption spectra of proteins and amino acids" including the effects of the peptide bond, the structural implications of spectral shifts (from those of the constituent amino acids), and the quantitative determination of aromatic amino acid residues by ultraviolet spectroscopy. A chapter by Sutherland gives a brief outline of infrared spectroscopy, discussing some of the principal infrared bands and their structural interpretation. Volume 8 (18) contains a review by Tiselius & Flodin of "Zone Electrophoresis" which discusses techniques in paper and in columns, and the limitations and errors to be encountered. Borsook discusses the thermodynamics of peptide bond formation, pointing out the considerably greater

ease of condensing peptides with each other than of condensing free amino acids. However, even under optimal conditions the yield by such a mechanism is small, and he goes on to discuss transpeptidations and energy-yielding mechanisms (high energy phosphate). An article by Putnam reviews bacteriophages and emphasizes fundamental problems and concepts in growth and reproduction. Finally, Volume 8 contains a concise yet comprehensive review on "naturally occurring peptides" by Bricas & Fromageot. The review emphasizes the isolation of peptides from bacterial, animal, and vegetable sources.³ It also accents the variety of linkages found in peptides in addition to the normal peptide link and makes one wonder as to whether such linkages occur widely in proteins also. The authors have discussed glutathione in particular detail, including its metabolic functions; the synthesis of the pteridyl peptides and their biological effects, including folic acid antagonism, are also considered.

GENERAL AND ORGANIC CHEMISTRY

Major achievements of the past year include the determination of the structures, followed by synthesis, of two posterior pituitary hormones, oxytocin and vasopressin. The structure of oxytocin was determined independently in two laboratories [Tuppy (19); du Vigneaud, Ressler & Trippet (20)]. The structure of vasopressin was reported by du Vigneaud, Lawler & Popehoe (21, 22). The proposed structure of vasopressin is supported by the findings of Acher, Chauvet & Fromageot (23). The striking similarity of oxytocin and vasopressin is immediately evident from the formulae shown in Figure 1. Replacing the leucine of oxytocin with arginine or lysine and the isoleucine with phenylalanine produces a vasopressin.

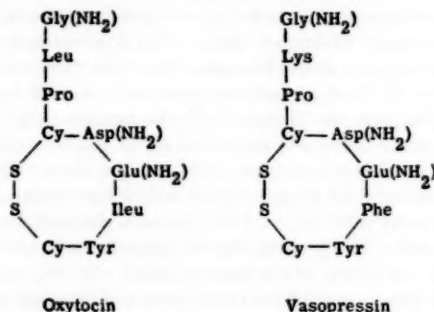
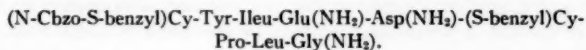


FIG. 1. Structure of oxytocin and vasopressin.

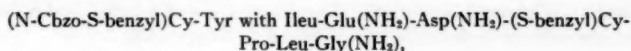
³ Peptides discussed include oxytocin, vasopressin, hypertensin, pepsin inhibitor, trypsin inhibitor, secretin, carnosine, anserine, glutathione, Borsook's peptide A, the pteroylglutamic acid family, polymyxins, licheniformins, actinomycins, subtilin, bacitracin, tyrocidines, gramicidins, nisin, D-glutamyl polypeptide, uridine diphosphate peptide.

Oxytocin.—The synthesis of oxytocin⁴ (24) hinges on the old finding that its disulfide bond is reversibly reducible (25). However, benzylation of the reduced compound produced an inactive product until it was found that the benzyl residues could be removed by sodium in liquid ammonia. Synthesis then might be achieved through the preparation of its S-dibenzyl-N-carbobenzoxy derivative.

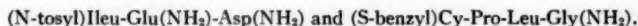
The structure of this S-dibenzyl-N-carbobenzoxy peptide was



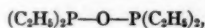
This was prepared by condensing



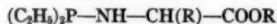
which was prepared from



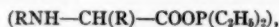
Tetraethyl pyrophosphite in peptide synthesis.—The pyrophosphite procedure of Anderson, Blodinger & Welcher (26) was used to couple these three peptides. The method employs tetraethyl pyrophosphite,



as a reagent. This reagent forms both amides



and anhydrides, presumably



with amino acids or peptides. These in turn condense with free amino or carboxyl groups to form new peptides. In the standard procedure equimolar amounts of carbobenzoxy or phthaloylamino acid (or peptide) and the amino acid ester (or peptide ester) are partially dissolved in diethyl phosphite. The mixture is then heated on a steam bath with 1.1 moles of tetraethyl pyrophosphite for 30 min. The peptide which is formed is precipitated with water. The preparation of a number of peptides in excellent yield is described. The development of this method through the synthesis of phosphite amides and phosphite anhydrides from diethyl chlorophosphite is described in the two preceding papers (27, 28).

A unique feature in the use of phosphite amides for peptide synthesis is that the amino group rather than the carboxyl group is activated. This is also true in the method of Goldschmidt & Lautenschlager (29) who prepared peptides in good yield from phosphorazo derivatives of amino acid esters of the type $\text{ROOC-CH(R)-N=P-NH-CH(R)-COOR}$. The reactions which

⁴ As a footnote to reference 21 they also report the synthesis of vasopressin by an analogous sequence of reactions.

Anderson *et al.* have employed are analogous to certain reactions which have been proposed for the biosynthesis of protein.

Thioesters in peptide synthesis.—The use of thioesters in the synthesis of peptides may have practical importance and, by analogy to some of the reactions of acetyl coenzyme A, biosynthetic mechanisms are also suggested.

Wieland & Schafer (30) prepared S-glycylthiophenol, as well as S-valyl-, S-leucyl- and S-methionyl-thiophenol. These thioesters react energetically with the amino groups of other amino acids or amino thioesters to form a number of di- and tripeptides which may be identified by paper chromatography. The mechanism of the reaction between amines and thioesters or thio-acids is discussed by Schwyzer (31). The preparation of thioalanine is described by Wieland & Sieber (32). The preparation of S-lactyl and S- β -oxybutyryl glutathione is described by Wieland & Köppe (33).

The synthesis of cyclic peptides.—This has been stimulated by the occurrence of cyclic peptides in many natural products. Winitz & Fruton (33a) have prepared cyclo-DL-phenylalanylglycylglycine by catalytic hydrogenolysis of carbobenzyloxy-DL-phenylalanylglycylglycine hydrazide. Bilek *et al.* (34) believe that cyclic peptides of high molecular weight are formed by the action of pyridine on carbonic anhydrides of glycine, phenylalanine, and their derivatives.

Other peptide syntheses.—These include the preparation of di-, tri-, tetra-, and penta-lysine by Waley & Watson (35, 36) and the conversion of diketopiperazine into a glycine polymer by Meggy (37). Frankel and co-workers (38, 39, 40) have synthesized poly-N-benzyl-DL-asparagine, poly-DL-serine, and poly-amino malonic acid. Schlogl, Wessely & Wawersich (41) describe poly-L-tyrosine. The poly-condensation of 11-amino-undecanoic acid has been studied by Vergoz (42).

The enzymatic synthesis of peptides.—This subject, we hope, will be covered in the review by Desnuelle in this volume. Fruton *et al.* (43) report the formation of polymeric products of glycyl-L-phenylalanylamide through the action of cathepsin. The products may be octa or decapeptides. Similar substances were obtained from glycyl-L-tyrosinamide, and L-alanyl-L-phenylalaninamide.

Removal of blocking groups in peptide synthesis.—This has been furthered by the study of Boissonnas & Preitner (44) on the relative stability towards several reagents of N-carbobenzyloxy, N-carbophenylloxy, N-tosyl, N-formyl, N-phthalyl, N-carbo-*p*-chlorobenzyloxy, N-carboallyloxy, N-carbo-*p*-tolylloxy, N-carbo-*s*-butyloxy, and N-carboethoxy groups attached to the amino group of α -amino acids. The first five may be selectively split one after the other, N-carbobenzyloxy by hydrobromic acid dissolved in glacial acetic acid, N-carbophenylloxy by catalytic hydrogenation, N-tosyl by sodium in liquid ammonia, N-formyl by methanolysis, and N-phthalyl by phenylhydrazine. The preparation of some N-carboethoxyamino acids is described by Vernsten & Moore (45).

However, synthesis is still a distant goal in the case of many peptides (and

all proteins) and work today centers rather on the elucidation of structure through the determination of chemical composition, chemical modification, and degradation. Studies on insulin, which is of a size intermediate between typical peptides and proteins, continue to lead the way.

The amino acid composition of insulin.—Beef, hog, and sheep insulins have been compared by Harfenist (46), following purification as the "A fractions" by Craig's counter-current distribution (47). All three have the same total number of amino acid residues and differ only in the relative numbers of threonine, serine, glycine, alanine, valine, and isoleucine residues. Among these latter it would appear that threonine can substitute for alanine, serine for glycine, and valine for isoleucine, since the sums of each of these pairs were also constant. The composition of beef insulin agreed precisely with that deduced by Sanger in his sequence studies. The other major component of beef insulin in counter-current distribution (B fraction) differed from the A fraction only in containing one less amide nitrogen. Harfenist emphasizes again the importance of studying different hydrolysis times for quantitative results, since at least four days in 5.7 *N* HCl at 110°C. was required for maximal isoleucine yields, whereas such conditions resulted in appreciable destruction of tyrosine.

The amino acid composition of serum albumin.—McClure, Schieler & Dunn (48) have reported a complete amino acid analysis of crystallized bovine serum albumin which differs markedly from the consensus of previous values, the yield of several amino acids being slightly less than that reported by Brand (49) and Stein & Moore (50). However, their yield of glutamic acid is 30 per cent greater. Incorporation of their high value for glutamic acid in Stein & Moore's analysis would result in more than 100 per cent recovery in terms of total nitrogen, which indicates a need for further investigation of this estimation. It is interesting to note that on the basis of their methionine and tryptophan analyses (assuming 4 and 2 residues per mole respectively) Dunn *et al.* were able to calculate a molecular weight of 65,000 for serum albumin, in agreement with recent physico-chemical estimations (see below) and distinct from the earlier estimates of 69,000. However, the hydrolytic losses of tryptophan have been shown to be so large as to vitiate its value in such estimates (51). In fact, accepting the losses found by Spies & Chambers, one may calculate that bovine serum albumin must contain 3 tryptophan residues instead of the 2 reported by Dunn. (This would also explain the reported (49) presence of only 0.7 tryptophan residues per mole of human serum albumin.) If the methionine analysis is as accurate as reported by the authors (± 2 per cent), this does give a method of calculating the molecular weight. However, it poses another problem in that, since serum albumin contains a single sulfhydryl grouping (52), the total number of sulfurs must then be an odd number, whereas Dunn and Brand both found 40 sulfurs per molecule of albumin ($M=65,000$) by elementary analysis.

Analytical methods.—Those of interest include Troll & Cannan's for the

quantitative determination of amino nitrogen by a modified ninhydrin method which gives identical color values for all of the α -amino acids containing primary amino groups, excepting tryptophan and lysine (53). Their modifications include the use of concentrated phenol solutions buffered with pyridine to stabilize the color, and the use of cyanide as a reductant. A modification of this procedure proved applicable for the determination of proline and hydroxyproline.

Modifications in the micro-Kjeldahl apparatus to permit more rapid distillation of ammonia (54), and the use of large amounts of mercuric oxide to speed digestion (55), have been reported, as well as a conductivity method for sulfur which utilizes lead instead of barium to precipitate sulfate (56). Johns & Marrack (57) have investigated the errors in the Morgan-Elson method for determining glucosamine.

Chemical modification of proteins.—Chemical modification, as a probe of protein structure, has been used by Mills to investigate the essentiality of the amino groups of insulin for biological function (58). For this purpose he chose an active polynitro hydrocarbon, 2,4,5-trinitrotoluene, which reacts similarly to DNFB, but at a slower rate. He was able to show that at pH 9.2 the terminal glycine reacts before the terminal phenylalanine or the ϵ -amino of lysine, and such substitution did not affect the biological activity. At pH 10.4 however, the lysyl residue reacted most rapidly, and biological function was soon destroyed. This difference in reactivity seemed reasonably attributable to the greater basicity of the ϵ -amino group. The loss of biological activity could not be directly attributed to the blockage of the lysyl residue since activity was sometimes completely destroyed when only a fraction of the single lysyl residue (M. of insulin: 6,000) had reacted. Similarly there was not sufficient reaction of terminal phenylalanyl or of tyrosyl hydroxyl to account for the inactivation, and, in fact, occasionally activity was fully retained when a significant fraction of each of the free amino groups had reacted. Consequently, the amino groups appear unessential for hormonal function, in confirmation of the studies of Fraenkel-Conrat who showed that acetylation of the amino groups did not affect insulin activity (59). The inactivation here observed must be a result of an unknown side reaction, perhaps oxidation, since Macheboeuf *et al.* have shown the disulfide groups of insulin to be remarkably labile towards oxygen at pH 10 (60).

Acetylation by acetic anhydride has been used by Green (61) in investigating the tanning reactions of collagen. In a series of partially acetylated samples, quinone binding decreased linearly with the decrease in free amino groups to the point where all had reacted. However, even after all of the amino groups had been acetylated, the collagen still retained a major portion of its affinity for quinone, and this was not altered by further acetylation. The effect of acetylation on formaldehyde binding was similar except that only a part of the amino groups appeared to be involved, and blockage of these almost completely destroyed formaldehyde binding. Tannic acid binding, however, was quite unaffected by any degree of acetylation.

In another article (62) Green has studied the effect of acetylation on binding of chromic ions by collagen. Again a linear relation between decrease in binding and increase in acetyl content was observed with approximately four acetyl groups blocking one chromium atom (this stoichiometry, however, depended on the nature of the chromium salt used). Here, also, complete acetylation only decreased the amount of binding by 30 per cent.

Saroff *et al.* have reinvestigated the methylation of bovine serum albumin by anhydrous acid methanol (63). They chose treatment of a suspension of the protein in methanol containing 0.1 *N* HCl for 20 hr. at room temperature, since this introduced methoxyls equivalent to the carboxyls originally present (by analysis, 94 per mole). However, this value did not correspond to a plateau in methylation since larger amounts, up to 120 methoxyls per mole of albumin, could be introduced by longer treatment. The authors conjecture that the additional methoxyls may have replaced amide groups, although no evidence of release of nitrogen is reported. In confirmation of Fraenkel-Conrat the authors found no evidence for methylation of nitrogen by this procedure, the methyl-imide analyses and infrared studies failing to reveal any $\text{CH}_3\text{-N}$ bonds (i.e., ± 3 groups per albumin molecule). These methylated derivatives showed a markedly smaller sedimentation constant, even when allowance was made for the large charge on the protein. This resembles the changes found in acid solutions of albumin (63a) where similar charges prevail and presumably represent an alteration in the shape of the molecule. Attempts to determine the isoelectric point of the methylated derivatives were thwarted by the rapid hydrolysis of methoxyl groups above pH 7.

Analysis of modified proteins.—Chemical modification, followed by hydrolysis and isolation of fragments containing the reacted groups, is becoming increasingly useful in studies of structure. A particularly fine example is the isolation by Schaffer, May & Summerson of O-phosphoryl serine from hydrolysates of chymotrypsin and of eel cholinesterase previously inactivated with diisopropyl fluorophosphate (64). Thirty per cent of the single radiophosphorus atom present on each chymotrypsin molecule appeared in a single band from a Dowex-50 column and followed a band which contained 50 per cent of the phosphorus as orthophosphoric acid. While the authors proved by control experiments that a phosphoryl serine could not arise from a reaction of diisopropyl phosphoric acid with serine during the hydrolysis, a similar exchange reaction with other diisopropyl phosphates cannot, of course, be excluded. Suspicion of such a reaction is strengthened by the findings of Wagner-Jauregg, O'Neill & Summerson (65) that diisopropyl chlorophosphate reacts preferentially with the amino group of serine and threonine, and of Plapinger & Wagner-Jauregg (66) that such N-phosphoryl derivatives readily rearrange to give O-phosphoryl serine and threonine respectively. Since no phosphoryl threonine was found in the hydrolysate of diisopropyl phosphoryl chymotrypsin, the presence, originally, of the phosphorus on either the amino or hydroxyl group of serine would appear probable. The above evidence thus suggests that a seryl residue may be involved in the catalytic function of this

enzyme and focuses attention on the "acyl shift" recently studied by Elliott (67). Elliott was able to hydrolyze silk fibroin specifically at the serine and threonine bonds by first rearranging seryl (and threonyl) peptide bonds to ester bonds with concentrated sulfuric acid, then blocking the amino groups thus liberated with acetic anhydride and gently hydrolyzing the ester bonds with alkali.

Chemical derivatives involving terminal amino acids.—These continue to be intensively studied because of their importance in determining amino acid sequences. Less reactive analogues of DNFB include the chloroderivative, derivatives containing active nitro groups (58), and 2,4-dinitrobenzene sulfonic acid (68). This last has the advantage of being water soluble, permitting homogeneous reaction with the protein without denaturation. Schroeder & LeGette (69), reporting on factors affecting the rate and yield in the reactions of DNFB with amino acids, point out that high pH values (i.e., Na_2CO_3 solutions) produce large amounts of dinitrophenol as a side product and that salts depress the reaction by salting out the reagent.

Ingram (70) has used a reductive methylation procedure (formaldehyde plus H_2 -Palladium catalyst) to convert terminal amino groups into dimethyl amino groups. Following hydrolysis of the protein, the derivatives resolve nicely in paper chromatography but are rather hard to identify. He has used acid-base dyes in conjunction with the ninhydrin reaction to differentiate them from the natural amino acids. Saroff (71) has found that the guanidino derivatives (by the action of methyl isourea) may be similarly used and identified on paper chromatograms by the Sakaguchi reaction.

In connection with the determination of terminal carboxyl groups by their reduction with metal hydrides following esterification, several new amino alcohols have been synthesized including arginol (72), histidinol (73), and cysteinol (74). However, most studies of terminal carboxyls have made use of carboxypeptidase. This may be made markedly more specific by pretreatment with diisopropyl fluorophosphate to inactivate any endopeptidases present (75).

Instances of peptides and proteins lacking terminal amino acids continue to grow. This situation appears to be the rule in the peptide antibiotics: licheniformin, actinomycin, bacitracin, tyrocidine, polymyxin (18). Ovalbumin, repeatedly found to lack a terminal amino group, is now also reported by Steinberg to lack a terminal carboxyl (76) since it is not attacked by carboxypeptidase⁵ until altered by other enzymes, such as the *B. subtilis* enzyme. Similarly the inactive proteolytic precursor, chymotrypsinogen, appears to contain no terminal amino acids and activation is accompanied by the splitting off of a peptide and the appearance of terminal amino acids (C-terminal plus N-terminal) in the enzyme (78). The activation of trypsinogen, on the other hand, appears to involve the splitting of a peptide from the N-terminal

⁵ Steinberg's earlier finding (77), that ovalbumin was attacked by carboxypeptidase, is now attributed by him to proteolytic impurities in the enzyme.

end only (79, 80). The peptide from trypsinogen is acidic (81) and that from chymotrypsinogen is basic (78).

The N-terminal amino acid of porcine pepsin was found to be leucine by the DNFB technique [Williamson & Passmann (82)]; the same workers (83) have found only alanine at the carboxyl end, both by carboxypeptidase and by lithium aluminum hydride reduction (isolation of alaninol).

Porter's amazing finding (84) that rabbit γ -globulin and rabbit antiovalbumin both contain the same N-terminal amino acid (alanine) has not proved to be general in other species. Van Vunakis reported seven terminal amino acids in human γ -globulin (Fraction II) in amounts of less than one mole of each per mole of protein (85). This has been partially confirmed by Putnam (86) and McFadden & Smith (87) who report 1 aspartyl residue and 2 (or 1) glutamyl residues, plus a fraction of a seryl residue, per mole of γ -globulin. Moreover, different fractions of human γ -globulin (II-1,2 vs. II-3 respectively) showed different contents of glutamyl (2 and 1 residues respectively) and of seryl end groups. γ -Globulins from myeloma patients gave still other results, differing from patient to patient (86). Bovine and equine γ -globulins contained five or more terminal amino acids in amounts of approximately 0.1 residue per molecule of protein (87).

Li & Ash (88) have estimated the terminal amino acids of hypophyseal growth hormone (somatotropin) by the DNFB technic and obtained 1 residue each of phenylalanine and alanine and 24 lysyl residues per molecule of protein [$M = 47,000$ from sedimentation and diffusion (88a)].

Mellon, Korn & Hoover (89) have similarly studied α and β -caseins and found terminal arginine and lysine. The α , ϵ -didinitrophenyllysine proved very unstable to the hydrolytic conditions (up to 82 per cent loss).

End-group analyses still do not yield unequivocal results, and quantitative measurements in terms of groups per molecule (with appropriate controls to aid in estimating yields) seem essential. Thus, one wonders whether the N-seryl residue reported above for γ -globulin represents another protein, or perhaps an "acyl shift" during the preparation of the protein. Traces of amino acids are also frequently adsorbed to proteins during fractionation and the albumin fraction (Fraction V) is heavily contaminated in this way [presumably because conditions necessary for the precipitation of albumin also markedly decrease amino acid solubility (90)].

An example of quite spurious end-groups [Schramm's (91) estimate of the N-terminal residues in tobacco mosaic virus] has been pointed out by Fraenkel-Conrat, Harris & Knight (93) who show that the hot trichloroacetic acid step in Schramm's procedure will hydrolyze peptide bonds, not only in tobacco mosaic virus but also in other proteins. They thus confirm their earlier report (92) of approximately 3000 C-terminal threonyl residues in this virus molecule, but now find less than 10 per cent as many N-terminal residues (93).

Even in serum albumin the number and kind of end-groups are still in dis-

pute. While the consensus of opinion from the DNFB technic is that human, bovine, porcine, and equine serum albumins contain but a single aspartyl residue (94, 95, 96), Dunn *et al.* (48) report finding aspartyl, histidyl, methionyl, and alanyl residues by the Edman method. These results may have been complicated by acid hydrolysis of sensitive peptide bonds, since they did not attempt to separate the thiohydantoin from free amino acids which might have been formed by the acid treatment.

Dunn *et al.* also report a large number of terminal amino groups in bovine serum albumin by microbiologically measuring decreases in amounts of specific amino acids following nitrous acid treatment of the intact protein. However, as pointed out by them, the analytical pitfalls in technique are serious, and we feel that these results should only serve to stimulate a reinvestigation of the terminal groups in serum albumin by more direct methods.

Arrangement of amino acids in peptides.—Amino acid order and arrangement in some peptide chains have been further clarified during the past year. There are two general approaches to this problem. In the first, partial hydrolysates are prepared, and a number of peptides are isolated and characterized. The most likely structure is then constructed by matching fragments containing overlapping sequences. The outstanding example is still Sanger's work on insulin for which the order has now been completely determined with Sanger & Thompson's reports on the sequence in the N-glycyl chain (97, 98). Further problems include, how the pairs of sulfur atoms are linked in disulfide bonds, the nature of the coiling of the peptide chains, and of course, the chemical mechanism of its physiological action, towards which the above sequence studies seem to offer no clue. Attempts to coil Sanger's chains into Pauling's helices provide geometrical difficulties in forming the disulfide bonds (99).

Other studies of sequence include the structures of oxytocin and vasopressin mentioned above, the isolation in appreciable yield of glycylalanylglycine from silk fibroin (100), the structure of the acidic peptide split from trypsinogen during its activation (Val-(Asp)₅-Lys) (78), and a series of peptides from clupein (101). The clupein results led Felix *et al.* to propose the structure: Pro-(Arg₄-M)_n-Arg₂ for this protamine, where M is a monoamino acid (101). However, Waldschmidt-Leitz (102) reports clupein prepared by Felix's method to be only 90 per cent pure. The impurity, separated by chromatography of the dinitrophenylated peptides, contained threonine and isoleucine, whereas the major fraction was free of these amino acids. The major component contained only proline as an N-terminal amino acid, and splitting by trypsin yielded DNP²-prolyl-seryl-arginine. Carboxypeptidase treatment suggested the carboxyl end sequence to be: seryl-alanyl-arginyl-arginine, in agreement with the work of Felix.

The above approach to sequence runs into difficulties when the number of fragments becomes too large, and hence efforts have been made to carry out a step-wise degradation of the peptide chain, beginning at either end. For this approach, it is necessary that: (a) the peptide resulting from the removal of

the terminal group be isolatable in a pure form, (b) that the initial protein or peptide have at least one and preferably only one terminal group, and (c) that the methods employed are applicable to all the structures which might be met in the course of degradation. Such structures may include β -amino acids [Christensen (103)], disulfide bridges, cyclic structures involving amides of carboxyl and amino side chains, and *D*-amino acids. These last might seem particularly serious in the case of the carboxypeptidase method. However, a still more serious limitation to the carboxypeptidase method lies in its relative rate of attack of a variety of terminal peptide bonds (78), which may be so great that terminal amino acids sometimes appear in pairs.

Step-wise degradation of peptides.—Edman's method, employing phenylisothiocyanate, is probably the most commonly used chemical procedure for step-wise degradation (104). In this method, after reaction with phenylisothiocyanate, the resulting phenylthiocarbamyl peptide is cleaved into a 3-phenyl-2-thiohydantoin and a peptide with one less amino acid residue. The phenylthiohydantoin may be hydrolyzed in barium hydroxide to the corresponding amino acid or identified directly by paper chromatography [Landmann *et al.* (105), Sjoquist (106)]. Dahlerup-Petersen (107) carried out the ring closure in 0.05 *M* citrate buffer at 60 to 70°C. and extracted the thiohydantoins into organic solvents as they formed. Edman (108) has suggested the use of anhydrous glacial acetic acid, saturated with anhydrous HCl, as a solvent for the cleavage of phenylthiocarbamyl peptides. The Edman technique, tested on insulin where the sequence is known, has failed after several steps either in the sense of criterion (a) above (successive incomplete reactions with the accumulation of by-products) or in the sense of (c) (arrival at a nonpeptide linkage such as a disulfide bridge (105)).

Another reagent for step-wise degradation is Reith & Waldron's 3,5-dinitro-4-dimethylaminophenyl isothiocyanate, which forms intensely colored thiohydantoins (109). However, the gain in chromatographic sensitivity so obtained is probably more than defeated by the loss in resolving power produced by the presence of the larger and more polar dinitrophenyl group. Leonis & Levy's method, involving a similar cyclization with carbon disulfide (110), has proven less promising than the phenylisothiocyanate method as its yields are still less quantitative.

Methods starting at the carboxyl end of the molecule are considerably less satisfactory as clean reactions have not been found. In one procedure (110) the peptide is heated with acetic anhydride and ammonium thiocyanate. The acylthiohydantoin thus formed is then hydrolyzed with dilute alkali. Baptist & Bull (111) found that the recovery by this procedure was far from quantitative. Rather than use acetic anhydride and ammonium thiocyanate to synthesize the thiohydantoin of the terminal amino acid, Kenner, Khorana & Stedman (112) have used the reagent diphenyl phosphoriso-thiocyanatide, $(\text{PhO})_2\text{PO-NCS}$, in solutions of acetonitrile or dimethylformamide. Diphenyl phosphate is eliminated and the desired thiohydantoin is produced in considerably improved yield.

PHYSICAL CHEMISTRY

Sedimentation analysis.—The large increase in the number of analytical ultracentrifuges over the past few years has resulted in a similar increase in comparable data permitting a more accurate evaluation of sedimentation constants. Shulman (113) has tabulated the data available for serum albumin and fibrinogen. Measurements by the same laboratory with two different types of machines (the Svedberg and the Spinco), with their rotors operating in hydrogen and *in vacuo* respectively, have accentuated generally unrecognized errors in determining the temperature of the sedimenting solution. The actual temperature of the cell has been determined by observing the melting (or solidifying) of diphenyl ether as the temperature of the rotor is changed, and extrapolating these data to the meniscus to correct for the effect of pressure on the freezing point (114). Gas friction apparently causes considerably more heating than was previously anticipated so that sedimentation constants obtained in hydrogen-jacketed machines may be more than 10 per cent too high when the temperature is determined by a thermocouple in the gas space (113).

On the other hand the vacuum-operated machine should give slightly low values since the rotor expands under centrifugation and causes about 1°C. of cooling (115). Shulman was not able to detect this temperature drop in his measurements of the freezing point of diphenyl ether in the Spinco machine. However, the temperature changes in the solution from compression should be in the opposite direction from those in the rotor and thermal equilibration may take an appreciable time. Shulman's results nevertheless indicate that both types of machines, when similarly calibrated with diphenyl ether, give almost identical results: the Svedberg type may still give about 2 per cent greater sedimentation constants, although this is hardly outside of the experimental error.

Cells modified to permit the formation of a boundary while the centrifuge is accelerating have been described (116, 117). These consist of reservoirs from which solvent is forced centrifugally through pin-holes to form a layer on top of the main body of solution. This can be done without excessive turbulence and produces a boundary in the middle of the cell which may be followed from the moment the ultracentrifuge reaches full speed; hence, the method is particularly suitable for studying small molecules which only slowly leave the meniscus of the conventional cell.⁶

Beams *et al.* have developed a magnetically suspended ultracentrifuge which is accelerated by an air turbine which may be disengaged at operating speed (119). This permits unusually constant temperatures and operating speeds (the rotor decelerates less than 0.1 per cent per day) and again is particularly suited for the study of small molecules ($M = 300$ to 10,000). A pulsed

⁶ Baldwin (118) has discussed the mathematical analysis of refractive index gradients which never leave the meniscus.

light system, permitting the optical analysis of several samples in a multiple cell rotor, has been reported (120).

Lewis, Green & Page (121) have now reported a broad survey on the ultracentrifugal flotation of serum lipoproteins in high density media (e.g., KBr solution of density 1.21). Lipoproteins, purified by flotation in this medium, are characterized in the ultracentrifuge by negative sedimentation constants ($-S_{1,21}$). All of the mammalian species examined had one or more of these rising components although there was a great deal of species variation both in the relative amounts and in the sedimentation constants of the several components. Individual variation within a species was also observed. The authors feel that there is a correlation between the electrophoretic mobility and the rate of flotation of these lipoproteins, the β components rising most rapidly, followed in order by the α_2 and α_1 electrophoretic components. In the case of lipoproteins from human serum they were able to show a direct quantitative comparison in the planimetry of ultracentrifugal and electrophoretic diagrams⁷ (123). While deviations outside of the normal range were frequently observed in atherosclerosis, no simple correlation with any ultracentrifugal component was evident (123).

Schjeide & Deutsch (124) report that removal of lipoproteins from the plasma of chick embryos by flotation in the ultracentrifuge reveals components with sedimentation constants not seen in whole serum. This may again indicate an interaction which is obscuring such components in the more complex whole plasma. However, it also suggests caution, since these components may be artifacts of the technique: i.e., the concentrated salt solution may have dissociated some lipoprotein complex.

Other physico-chemical techniques.—Several other physico-chemical techniques have undergone improvements during the year. Longworth has described an electrophoretic apparatus for small volumes (125) and Randrup has described a sampling technique for determining mobilities of biologically active components in the Tiselius apparatus (126). Ogston & Stanier (127) have described a Couette viscosimeter which may be adapted for the measurement of streaming birefringence, and Smithies has described a dynamic osmometer giving high precision on very small volumes of solution (128). Zahn has devised a continuous extractor for protein fractionation (130).

Protein precipitation.—The use of polyelectrolytes as protein precipitants has been extended by Berdick & Morawetz (131), who have studied the use of several synthetic polyacids in the separation of mixtures of catalase and serum albumin, and of catalase and hemoglobin.

These procedures employ polyelectrolytes to displace, competitively, the normal protein-protein interaction, which can be done at very small polyelec-

⁷ Harrington & Schachman, by analyzing synthetic mixtures of viruses, have pointed out again that the area in the ultracentrifugal diagram is not necessarily proportional to the amount of the component present (122).

trolyte concentrations because of the latter's high charge density. Under proper conditions of pH, some protein-polyelectrolyte complexes are very insoluble, and the protein may be recovered by precipitating the polyelectrolyte with a suitable counter-ion. [Soluble complexes also form and have been used by Singer *et al.* for separations by electrophoresis-convection (132).]

While it has been possible to find satisfactory conditions for affecting separations of all proteins tested, the cases studied are very limited (131, 133, 134), and no pattern has emerged for predicting probable conditions for further separations. Some of the interactions have proven remarkably strong, extending far outside the pH range expected for electrostatic interaction, and ternary complexes of polyelectrolyte with both proteins have appeared under conditions where no precipitation by the polyelectrolyte occurred in the presence of either protein alone. Nevertheless, no evidence of denaturation has been observed in these procedures (131, 133), and in the case of catalase the enzyme-polyacid complexes themselves were catalytically active.

Chromatographic separations.—In chromatography of small peptides, the paper of Dowmont & Fruton (135) on the separation of peptides by ion exchange on Dowex-50 remains one of the best sources of reference. Schroeder & Honnen have studied the correlation between the structures of some di- and tripeptides and their chromatographic behavior on silicic acid-celite (135a).

The use of columns of ion exchange resin has not been limited to the separation of small peptides and amino acids, but has been extended to larger peptides and proteins. The theory of the separation of neutral proteins on ion-exchange resins is discussed by Boardman & Partridge (136). They point out that both charge and surface configuration of proteins are involved in such separations. As examples, they describe the separation of lysozyme from cytochrome-*c*, and of sheep fetal carboxyhemoglobin from bovine carboxyhemoglobin on the carboxylic ion exchange resin, Amberlite IRC-50. When cytochrome-*c*, prepared from heart muscle by conventional procedures, was chromatographed, 75 per cent of the material measured at 548 m μ was recovered apparently chromatographically pure.

Tallan & Stein (137) have identified three chromatographically distinct components of lysozyme on IRC-50. Hirs, Moore & Stein (138) also used IRC-50 to chromatograph pancreatic ribonuclease on a preparative scale. The procedure of Hirs *et al.* compares favorably with that of Martin & Porter (139) who employed partition chromatography on kieselguhr for separating two components in pancreatic ribonuclease, both possessing ribonuclease activity. Porter now feels that this second component represents a complex of ribonuclease with another protein (140).

Porter's method of partition chromatography uses the two phases: aqueous salt solutions and cellosolves, one phase being immobilized on kieselguhr columns (140). His results on crystalline insulin do not show the heterogeneity reported by Craig & Harfenist (47). However, whether this represented a poorer resolving power of Porter's system, or better insulin, could not be decided. In this regard it should be mentioned that Timasheff *et al.* (141)

have also observed heterogeneity in insulin by electrophoresis which, however, did not correspond to that found by Craig.

Merely by varying the ionic strength of aqueous phosphate buffers, Polis & Shmukler (142) were able to chromatograph lactoperoxidase on tricalcium phosphate and silicic acid-celite columns. Final preparations showed a molecular weight of 82,000. Salt fractionation on columns is also discussed by Schwimmer (143).

The partition chromatography of proteins on filter paper with phenol-water systems is described by Grassmann & Deffner (144). Chromatograms of serum albumin, γ -globulin, and clupein, as well as chromatograms of proteolytic digests of collagen with pepsin and chymotrypsin, are illustrated. Filter paper strips impregnated with sodium sulfite to produce essentially a paper strip cation exchanger have been used by Larson, Sternberg & Peterson (145) in their study of the antibiotic streptolin; 75 per cent ethanol containing sodium chloride was used as a developer.

The study by Agren & Glonset (146) of the phosphopeptones of casein is instructive for the number of different techniques employed, including ionophoresis and chromatography on carbon, Dowex-2, Dowex-50, and paper powder columns, as well as for a method for detecting phosphopeptones on paper.

A chromatographic procedure for the separation of proteins implies interaction between the protein and the material with which the chromatogram is prepared. Such interaction may lead to denaturation. Electrophoretic techniques in which paper, starch, or other supposedly inert material is used as a support for the buffer during an electrophoretic fractionation permit the separation of complex mixtures, such as serum proteins, to the extent that they show different electrophoretic mobilities. Adsorption in electrophoretic procedures is usually undesirable. The reader is referred to the review of Tiselius & Floden, mentioned above (18), for a full discussion of the potentialities of zone electrophoresis. Specific examples of the use of zone electrophoresis will be found in a number of the references in the present review.

SPECIFIC PEPTIDES AND PROTEINS

Tissue peptides.—These are still largely limited to glutathione and occasional specialized structures such as carnosine and anserine, no appreciable quantities of other peptides having been found in tissue. Christensen & Riggs (147) have studied the amino acids and peptides of chicken oviduct and find that almost all of the free peptide nitrogen is attributable to glutathione. However, unidentified spots occur with some frequency during paper chromatography of tissue filtrates. Some of these may eventually be recognized as specific peptides (148).

While it is very difficult to demonstrate any peptides in tissue, there are certain indications from isotopic data that they may play a role in protein synthesis. [Francis & Winnick (149); Steinberg & Anfinsen (150); Peters (151)]. If such intermediates exist they are probably closely associated with

the macromolecules carrying out the synthesis of protein, and their isolation may require special chemical techniques. Dounce (152) has published some interesting speculations concerning the role of pentose nucleic acid in protein synthesis.

Urinary peptides.—Stein, in his chromatographic analysis of normal urine on Dowex-50 columns has sought urinary peptides. He estimates that about 1 gm. of free amino acids and 2 gm. of conjugated amino acids are excreted per day (153). Glycine, glutamic acid, and aspartic acid comprise the major portion of the conjugated amino acids, while the amounts of proline, cystine, serine, threonine, and valine are also markedly increased by hydrolysis. Stein points out that a 2 or 4 per cent cross-linked Dowex-50 resin, rather than the conventional 8 to 12 per cent, would be more desirable for the actual separation of peptides. The protein composition of normal human urine has been discussed by Rigos & Hiller (154).

Carsten (155) carried out a preliminary desalting of human urine on ion exchange columns prior to chromatography on the acid form of Dowex-50 with HCl as an eluent. He found several small peptides to be present. Bode, Becker & Böhle (156), by comparing paper chromatograms of chloroform extracts of human urine, before and after hydrolysis, have also found conjugated amino acids. Slater & Kunkel (157), who carried out filter paper electrophoresis on the proteins of pathological urine, found that urine, following dialysis, often contained low molecular weight components not usually present in plasma. They speculate that these may represent low molecular weight proteins produced under the stimulus of protein depletion, which are rapidly cleared by the kidney.

Inasmuch as many of the protein systems will have been treated in detail in the second volume of *The Proteins* (2), the remainder of this review will be concerned with isolated developments with regard to specific peptides and proteins.

The adrenocorticotrophic hormone (ACTH).—As isolated, this hormone may be an active fragment of a larger pituitary hormone, and, furthermore, active fragments of different size may exist. Thus White & Fierce (158) isolated, without preliminary hydrolysis, a fraction which they called corticotropin-A. This was purified by adsorption on oxycellulose followed by chromatography on Amberlite XE-97 and further purified by counter-current distribution (159). It then appeared to be homogeneous by these techniques.

Corticotropin-B has been obtained by Folker's group, from pituitaries treated with acid and pepsin, by a similar combination of chromatographic and counter-current distribution (160, 161, 162). It also appears to be homogeneous and has about twice the activity of corticotropin-A. White & Fierce have studied the effects of pepsin, and pepsin followed by acid, on unhydrolyzed partially purified corticotropin, observing the appearance of new components consistent with the formation of corticotropin-B from A by hydrolytic changes. In fact, two active degradation products were observed. While

the formation of corticotropin-B from A by a simple cleavage into two approximately equal fragments (one inactive) would be suggested by the relative activities of the two products, the instability of corticotropin, resulting in inactive molecules of similar physical properties, makes such an assumption questionable.

Nevertheless, that some splitting must occur is indicated by White's studies (163) of the action of pepsin on corticotropin-A, which liberated the peptide: pro-leu-glu-phenylalanine. That this was a terminal sequence was indicated by the liberation, in sequence, of phenylalanine, glutamic acid, and leucine from corticotropin by carboxypeptidase. Terminal proline is not split by this enzyme, thus confirming this sequence. Folker's group reports corticotropin-B to contain no leucine (164). In view of the relatively large amount of leucine in corticotropin-A, the hypothesis of peptic action being limited to the removal of this terminal sequence would require either a much smaller molecular weight for corticotropin-B than the 5000 estimated from sedimentation and diffusion (164) or the presence of several similar chains in the corticotropin molecule.

The N-terminal sequence of corticotropin-A, determined both by the Sanger and the Edman techniques, is seryl-tyrosyl- (165). Furthermore, hydrolysates of the DNP-hormone yield no free serine. This again suggests that either several identical end-groups are present or that the molecular weight is much smaller than generally supposed, since Folkers' group (164) found three to four serines per molecule ($M = 5,000$).

Finally, it might be added that no evidence for a prosthetic group has been obtained, and more than 87 per cent of the hormone (but 99 per cent of its nitrogen) has been accounted for in terms of its constituent amino acids (164). However, as already mentioned, the hormone shows a marked tendency to lose activity during purification and this lability is counteracted by thiols, although the hormone contains no cysteine or cystine (164). Thus the reason for this lability is unknown.

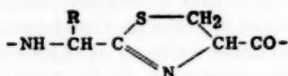
Peptide antibiotics.—The greatest advances reported on the peptide antibiotics, since Bricas & Fromageot's current review (18), have been in the chemistry of the bacitracins. Newton & Abraham (166) and Craig *et al.* (166a), both using counter-current distribution, but with different solvents, have isolated a major component (Bacitracin-A) from commercial bacitracins. The impurities consist of related but chemically distinguishable components.

Craig has determined the molecular weight by his partial substitution method, obtaining a value of 1470 (167); this agrees with a minimum molecular weight calculated from sulfur analysis of 1450 (166a) and also with Longsworth's estimate from diffusion. Complete amino acid analysis (168) indicated the presence of two L-isoleucine residues and one each of L-leucine, cysteine, histidine, lysine, and aspartic acid. One D-aspartic acid residue was also present as well as one residue of D-glutamic acid, one of D-ornithine, and one ammonia. However, these analyses only account for about 90 per cent

of their proposed molecular weight for this substance, and they therefore assume that some unknown residue (which must contain nitrogen) is also present (168).

Further features of interest in the molecule are the presence of only two free carboxyl groups by titration (169) and of two free amino groups (reacting with DNFB) (167). One of these amino groups appears to be ornithine (167, 169) and the other leucine or isoleucine (169). The imidazole group also reacted with DNFB (169). Therefore, a cyclic structure involving the ϵ -amino of lysine and one of the free carboxyls seems required. (Unlike several cyclic antibiotics, this peptide contains 11 rather than 10 amino acids.) The presence of the ammonia as an amide on another carboxyl then reduces the number of free carboxyls to two as required.

The most paradoxical behavior of this peptide lies in its sulfur, which has the oxidation state of cysteine, but gives no nitroprusside test until after treatment with 0.5 *N* HCl at 100°C. for 20 min. (169). This treatment also liberates the ammonia and destroys the absorption maximum of bacitracin at 252 m μ . Alkali treatment, on the other hand, liberates ammonia without affecting the sulfur reactivity so that these two reactivities appear unrelated. Newton & Abraham further showed that hydrogenolysis with Raney nickel converted the cysteine to alanine, in which the amino group was now free (i.e., reacted with DNFB to give DNP-alanine upon hydrolysis). Their tentative, and very appealing, explanation for all of these phenomena is that bacitracin contains a thiazoline ring structure:



This structure has been postulated by Linderstrøm-Lang & Jacobsen (170) as an explanation for masked thiols in proteins. A further study of the hydrolytic cleavage of such rings has recently been made by Crawhall & Elliott (171).

Natural poly-D-glutamic acid.—This acid has been shown to be linked primarily through the γ -carboxyl since the Hofman and Curtius degradations of this material produce formyl propionic acid (172, 173). On the other hand, similar degradation of the synthetic polypeptide of L-glutamic acid, in which the α -carboxyl is in the peptide link gives α , γ -diaminobutyric acid as expected (174).

The hyperglycemic factor of insulin.—Studies on insulin, including purification (47, 140, 141), composition (46), structure (97, 98), and reactive groupings (58, 59), have been mentioned above. However, probably the most interesting development is the isolation in crystalline form of the hyperglycemic, glycogenolytic, factor frequently associated with it.

The hyperglycemic factor was purified by Behrens *et al.* (175) from an "amorphous fraction obtained during the commercial purification of insulin."

Boser & Mohnike (176) have purified it, following extraction from the pancreas with hot (70°C.) aqueous cysteine solution. The use of cysteine to inactivate the insulin was also utilized in the extraction procedure of Foa *et al.* in which liquid ammonia was used as a solvent (177). However, none of these workers has yet published the details of their method.

Some of the properties of this factor, as discussed by Boser (178), are hard to reconcile with its usual concurrence with highly purified insulin. Thus he reported it to be a nucleoprotein (10 per cent ribose, 3 per cent phosphate, and 4 per cent adenine-like base). Moreover, it was polydisperse on electrophoresis.

Thymus peptide.—A thymus peptide with unusual inhibiting power for the growth of mycobacteria (such as tubercle bacillus) has been isolated by Hirsch & Dubos (179). It is a histone-like basic peptide whose action may involve an affinity for sulfate ions since it is neutralized by an equal weight of magnesium sulfate, and magnesium chloride is ineffective. However, ten times as much sodium sulfate as magnesium sulfate is required to produce equal neutralization which suggests that more complex phenomena may be involved.

Hemoglobins.—Hemoglobins with different chemical properties are inherited in humans. The identification of these different hemoglobins in terms of their solubility, resistance to alkali, and electrophoretic mobility has been reviewed by Itano (180), and a system of nomenclature for these (hemoglobins A, F, S, C, D) has been adopted (181). Their identification by paper electrophoresis is also possible (182).

The difference in oxygen affinity of adult and fetal human blood appears not to be attributable to differences in the hemoglobins, since Wyman *et al.* (183) have shown that fetal and maternal hemoglobin solutions, which show quite different oxygen affinities, appear identical after dialysis against a common buffer. This suggests that a dialyzable constituent is involved and brings to mind Riggs' report that the oxygen affinity of hemoglobin is affected by glutathione (184).

Lewis (185) has shown that the cleavage of hemin from four different proteins (hemoglobin, myoglobin, catalase, and peroxidase) in acid acetone is characteristic of the protein and of the acid used. Hydrochloric acid is effective at higher pH values than sulfuric acid, and the critical pH for cleavage varies from protein to protein. On the other hand all four proteins were similarly cleaved by sulfuric acid at pH 2.1. The kinetics of the acid-splitting of peroxidase indicate that two protons are involved [Maehly (186)].

The magnetic properties of the peroxide complexes of peroxidase, myoglobin, and catalase have been studied by Theorell & Ehrenberg (187). George & Irvine have postulated the presence of a higher oxidation state of iron (Fe^{4+}) to explain the action of hydrogen peroxide and other oxidizing agents on metmyoglobin (188, 189).

Collagen.—This extracellular fibrous protein is one of the principal con-

stituents of skin, tendon, bone, fascia, and the connective tissue which occurs in practically every organ of the body. It is characterized by its unique chemical composition and x-ray diffraction pattern. Each 100 gm. of collagen contains approximately 14.5 gm. of hydroxyproline, 1.2 gm. of hydroxylysine, 14.6 gm. of proline and 26.9 gm. of glycine.*

The hydroxyproline of collagen hydrolysates is apparently formed, from proline, by a route that does not pass through free hydroxyproline. Stetten (190) found that N^{15} -labeled hydroxyproline was poorly incorporated into collagen. N^{15} -labeled proline, on the other hand, contributed significant amounts of N^{15} to the hydroxyproline of collagen.

Collagen is the only protein which has been found to contain hydroxylysine. This amino acid appears to be formed from lysine [Sinex, Van Slyke & Hollister (191)]. The observation by Gordon (192), confirmed by Astrup *et al.* (193), that free hydroxylysine phosphate is present in a number of tissues, raises the question of the possible role of phosphate esters of hydroxylysine in collagen synthesis.

Grassman & Hormann (194) were unable to demonstrate any N-terminal amino acid residues in collagen by the DNFB technique or any C-terminal residues by thiohydantoin formation from NH_4CNS .

There does not seem to be any information available on the presence or absence of terminal groups in the protein initially prepared from skin by Tustanovskii *et al.* by extraction with 0.1 M citrate buffer of pH 3.5 (195, 196). Chernikov (197) found that this protein has an amino acid composition similar to collagen, although no values are reported for hydroxyproline or hydroxylysine. The protein aggregates into fibrils which exhibit certain features found in native collagen, but which also show an unusually large periodicity of 2000 to 2600 Å (198, 199, 200).

Some thirty peptides have now been isolated from partial hydrolysates of collagen (201, 202, 203), and by combining information on probable amino acid sequences with x-ray diffraction data some fairly detailed models of the structure of the collagen molecule have been proposed. Some of these were reviewed by Randall, Fraser & North (204).

Blood clotting.—Blood clotting is assuming the aspects of a respectable chemical process as further components of the system become isolated in a relatively pure form. The appearance of peptides in the clot liquor (205) and the presence of terminal amino groups in fibrin, different from those found in fibrinogen (206, 207), indicate that thrombin is a proteolytic enzyme. This enzyme has been reported to attack arginyl esters (208). Seegers & Alkjaerisig state that it can both activate and destroy prothrombin autocatalytically (209). Its action on fibrinogen produces at least two peptides (207), and it is not species specific, bovine thrombin clotting human fibrinogen and vice versa, even though these two fibrinogens contain different end-groups (210).

Bovine prothrombin, purified by Seegers, has been physico-chemically

* Tristram has tabulated the compositions of several collagens and elastins (2).

characterized by Lamy & Waugh (211), who calculate from sedimentation and diffusion a molecular weight of 63,000 and an axial ratio of 3.7. (They calculate an axial ratio of 3.4 from viscosity measurements.) These values indicate that the molecule closely resembles serum albumin in size and shape.

Seegers' bovine prothrombins are reported to be homogeneous when tested by the phase rule (constant solubility with excess saturating body) (211), and a single component is also observed in electrophoretic (212) and in ultracentrifugal (211) studies. However, human prothrombin of activity equal to the best bovine prothrombin was heterogeneous in the ultracentrifuge (209), suggesting either that human prothrombin has a higher specific activity than bovine or that both preparations are still impure. Bovine thrombin, prepared by the activation of prothrombin with concentrated sodium citrate, was definitely heterogeneous in electrophoresis (213).

Plasminogen, the fibrinolytic pro-enzyme of plasma, has been prepared electrophoretically homogeneous (214) and also has been crystallized (215). Kline's procedure (215) makes use of the extreme stability of plasminogen towards acid and alkali: Following acid extraction (0.05 N H_2SO_4) of the plasminogen from Cohn's fraction III, it was adjusted to pH 11 for three min. Upon neutralization to pH 5.3, followed by readjustment to pH 2, most of the protein could be removed as a gel, and the now partially purified pro-enzyme was readily further purified by fractional precipitation at pH 6. Such material, possessing an activity 425 times that of plasma, crystallized from salt-free solutions at pH 8 without further enhancement of the activity.

The specificity of calcium for blood clotting is now equalled by its role in complement (216). Its presence at 2×10^{-4} M concentrations proved necessary for complement fixation to specific precipitates, and no other ion could be substituted. In immune hemolysis, magnesium, as well as calcium, was necessary. However, specificity for magnesium was less absolute, since nickelous or cobaltous ions could be substituted.

AMINO ACIDS

The list of naturally occurring amino acids continues to grow. This is, in part, the result of extensive application of paper chromatography and ion exchange techniques to studies of the biological transformations of amino acids, the determination of the free amino acids of tissue, and the structure of antibiotics.

δ -Aminolevulinic acid.—This acid ($NH_2 \cdot CH_2 \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH$) has been synthesized and demonstrated to be an intermediate in porphyrin synthesis by Shemin & Russell (217). This would appear to be a result of brilliant deductive reasoning by the organic chemist, stemming from the earlier work which showed that glycine and "active" succinate were utilized in porphyrin synthesis. Figure 2 demonstrates that δ -aminolevulinic acid provides all of the atoms for the "precursor pyrrole" and for the porphyrin itself. Moreover, the "precursor pyrrole" has recently been isolated and identified in a pathological urine (218, 219, 220). Shemin's discovery seems

to be of the greatest importance since it would appear generally applicable to porphyrin synthesis in all plants and animals, the various known porphyrins resulting from subsequent alterations of the side chains. Moreover Shemin further postulates the role of δ -aminolevulinic acid to be that of a key intermediate in various other metabolic pathways, being geared to the Krebs cycle.

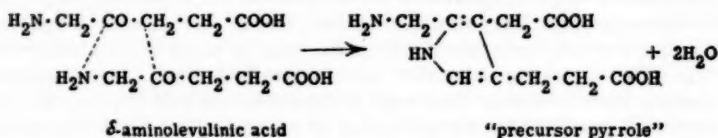


FIG. 2. Relationship of δ -aminolevulinic acid to "precursor pyrrole."

Monoamino-monocarboxylic amino acids.—The new monoamino-monocarboxylic amino acids include 5-hydroxytryptophan, synthesized by Ek & Witkop (221). Udenfriend, Clark & Titus (221a) report that the venom glands of the tropical toad *Bufo marinus* contain a substance indistinguishable from this by paper chromatography. As a result of the obvious relationship between 5-hydroxytryptophan and the naturally occurring pressor amine, serotonin (5-hydroxytryptamine), the natural occurrence of 5-hydroxytryptophan in other organisms must be considered a distinct possibility.

C^{14} -L-lysine is converted to C^{14} -L-pipecolic acid (piperidine-2-carboxylic acid) by the rat [Rothstein & Miller (222)]. They believe that pipecolic acid is an intermediate between lysine and aminoadipic acid. The formation of pipecolic acid from lysine also proceeds in the green bean (*Phaseolus vulgaris*). This was demonstrated by Grobbelaar & Steward (223) with C^{14} -labeled lysine. Morrison (224) isolated pipecolic acid from the leaves of white clover.

Extracts of *Neurospora crassa* were found to synthesize proline from glutamic semialdehyde [Fincham (225)], thus providing additional evidence that the latter substance may be the intermediate in the synthesis of proline from ornithine. A synthesis of the diethylacetal of glutamic- γ -semialdehyde is described by Good & Mitchell (226). Rat liver is reported to form α -amino-*n*-butyric acid from threonine [Lien & Greenberg (227)].

Guanidinobutyric acid has been demonstrated in certain marine invertebrates where it is presumably formed from the δ -guanidino- α -ketovaleric acid which arises from the action of L-amino acid oxidase on arginine [Robin (228)].

Boulanger & Osteux (229) have observed that incubation of homogenates of intestinal mucosa with pyruvic acid leads to the formation of a small quantity of a substance which is oxidizable by D-amino acid oxidase and which they assume to be D-alanine.

Dicarboxylic amino acids.—The newer dicarboxylic amino acids include

α -formamidino-glutaric acid isolated from digests of L-histidine or urocanic acid with cat liver preparations as reported by Borek & Waelsch (230).

While the natural occurrence of L- α -amino adipic acid seems rather well established, the D-isomer has not been previously reported. This has now been obtained from the antibiotic, cephalosporin-N, by Newton & Abraham (231). It is now apparent that α,ϵ -diaminopimelic acid has a fairly wide distribution in bacteria. Salton (232) found this amino acid in the cell walls of *B. subtilis*, *Escherichia coli*, and *Salmonella pullorum*. The preparation of 250 mg. of α,ϵ -diaminopimelic acid from 2.25 liters of *E. coli* culture fluid is reported by Work & Denham (234).

There is continued interest in the amino acid found in the groundnut by Done & Fowden (235), γ -methylene glutamic acid, $\text{HOOC}\cdot\text{C}(\text{CH}_2)\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{COOH}$. Its enzymatic decarboxylation has been studied by Fowden & Done (236). The corresponding 4-methyl glutamic acid has been synthesized [Fillman & Albertson (237); Smrt & Sorm (238)].

Basic amino acids.—Among the newer basic amino acids, isolysine has been found in the antibiotics streptolin (239) and viomycin (240), and its structure has been confirmed by synthesis to be β,ϵ -diaminocaproic acid, $\text{NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{COOH}$ [Van Tamelen & Smismann (241)].

While collagen is the only protein known to contain hydroxylysine, As-trup, Carlström & Stage (193), in confirmation of Gordon (192), have found the phosphate ester of hydroxylysine as a free amino acid in a number of the tissues of adult and embryonic ox, while Barbier & Lederer (244), have reported a hydroxylysine phosphatide in *Mycobacterium phlei*. The hydrolysis of gelatin or collagen gives two diastereoisomers of hydroxylysine [Van Zyl, Van Tamelen & Zuidema (245)]. These optical antipodes of hydroxylysine and allohydroxylysine have now been prepared and characterized by Fones (246). A synthesis of hydroxylysine-6- C^{14} has been achieved by Lindstedt (247), thus providing a useful tool for further study of this interesting amino acid.

The biosynthesis of arginine from citrulline and aspartic acid leads to the formation of argininosuccinic acid: $\text{HOOC}\cdot\text{CH}(\text{NH}_2)\cdot(\text{CH}_2)_3\cdot\text{NH}\cdot\text{C}(\text{CH})\cdot\text{NH}\cdot\text{CH}(\text{COOH})\cdot\text{CH}_2\cdot\text{COOH}$. This substance has now been isolated and synthesized by Ratner, Petrack & Rochovansky (248). An argininosuccinase prepared from jackbean meal has been used by Walker (249) to prepare a new amino acid, canavaninosuccinic acid: $\text{HOOC}\cdot\text{CH}(\text{NH}_2)\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{O}\cdot\text{NH}\cdot\text{C}(\text{:NH})\cdot\text{NH}\cdot\text{CH}(\text{COOH})\cdot\text{CH}_2\cdot\text{COOH}$.

Amino acids which contain sulfur.—This group includes 2-aminoethanesulfinic acid which Awapara & Wingo (250, 251) have observed, radio-labelled, on paper chromatograms of the free amino acids of rats which had been fed S^{35} -cysteine. They adopt the view of Bergert, Chatagner & Fromageot (252) that 2-aminoethanesulfinic acid is the primary intermediate between cysteine and taurine.

Thoai *et al.* (253) found guanidinotaurine, $\text{H}_2\text{N}\cdot\text{C}(\text{:NH})\cdot\text{NH}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{SO}_3\text{H}$ in a polychaete worm (*Sebellaria alveolata*) and a gephyrean

worm (*Phasiolosoma elongatum*). They believe that it is present in these organisms as phosphoguanidinotaurine.

The active methyl donor formed enzymatically from methionine and ATP is now thought to be S-adenosylmethionine [Cantoni (254)]. A new sulfur-containing amino acid has been isolated from cat urine by Westall (255). He assigns the provisional structure, $\text{HO}_2\text{C} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{S} \cdot \text{C}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{CH}_2\text{OH}$. A cat may excrete 100 to 120 mg. of this amino acid in a day.

The two antibiotics, subtilin and nisin, contain the amino acid lanthionine [Alderton & Fewold (256); Berridge, Newton & Abraham (257)] previously found in alkaline hydrolysates of wool where it is considered to be an artifact. In addition nisin and subtilin contain a β -methylanthionine, $\text{HOOC} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{S} \cdot \text{CH}(\text{CH}_3) \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH}$ [Newton & Abraham (258); Alderton (259)]. Alderton has synthesized this compound and determined the configurations about the α -carbon atoms.

Synthesis of amino acids.—The syntheses of amino acids under primordial conditions has been simulated by Miller (260) who circulated CH_4 , NH_3 , H_2O , and H_2 past an electric discharge. Glycine, α -alanine, and β -alanine were formed from the free radicals produced. They were identified by paper chromatography.

The use of inert solvents such as toluene or benzene in the sodium hydride condensation of ethyl halides with diethyl formamidomalonate or ethylacetamido cyanoacetate is advocated by Shapira, Shapira & Dittmer (261) on the basis of superior yields and convenience.

Birkofer & Storch (262) have synthesized β -aminopimelic acid, β -alanine, β -aminobutyric acid, β -amino-*n*-valeric acid, β -amino- γ -phenylbutyric acid and β -lysine by reacting hydrazoic acid with the appropriately substituted γ -keto ester. The reaction is illustrated by the following equation:



β -Aminobutyric acid may be conveniently and economically prepared from pyrrolidone (γ -butyrolactone) [Thompson, Pollard & Steward (263)]. Two stereoisomeric forms of β -hydroxy-DL-valine, homologues of DL-threonine and DL-allothreonine, have been prepared by Buston, Churchman & Bishop (264). *Erythro*- and *threo*- α -amino- β -hydroxy stearic acids have been prepared by Carter, Harrison & Shapiro (265).

Four new syntheses of tryptophan have been reported by Holland & Nayler (266, 267). Two of these start from ethyl- α -aceto- β -3-indolylpropionate, one from ethyl- α -cyano- β -3-indolylpropionate and one from 2-thio-5-thiazolidine. Reid & Schiller (268) have described the preparation of some quinolylalanines which might be considered as analogues of tryptophan.

The antibiotic chloroamphenicol, *threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol, has aroused interest in the synthesis of substituted phenylserines. *Threo*- β -phenylserine has been known for some time (269). However, the preparation and properties of *threo*- and *erythro*-*p*-nitrophenylserine have been the subject of some controversy, which now appears to

have been resolved (Holland, Jenkins & Nayler (270); Ehrhart (271); Carrara, Pace & Cristiani (272); Viscontini & Fuchs (273); Bolhofer (274)).

The reaction between benzylamine and maleic anhydride has been employed by Frankel, Liwischitz & Amiel (275) to synthesize DL-aspartic acid and DL-asparagine. The synthesis of aspartic acid-2,3- C^{14} - N^{15} is described by San Pietro (276).

A synthesis of valine-2- C^{14} from glycine-2- C^{14} via the 2-phenyl-4-isopropylpyridine oxazolone-5 is described by Adams & Tolbert (277). The synthesis of N^{15} -L-phenylalanine is described by Baldrige, McCarville, & Sendroy (278), while a small scale synthesis of 2,5-dihydroxyphenyl-DL-alanine is described by Shulgin (279).

Histidine-2- C^{14} has been synthesized by Sprinson & Rittenberg (280) and by Toporek, Miller & Bale (281) from $KSC^{14}N$ and γ -keto-L-ornithine, and by Wolf (282) from α - C^{14} -ethyl acetamidocyanoacetate.

Syntheses of lysine labelled in the 1st, 2nd, and 6th carbon atoms respectively have been reported. Lindstedt converted the bisulfite addition compound of γ -acetamido- γ' , γ' -dicarbethoxybutyraldehyde to the C^{14} -cyanohydrin. The cyanohydrin was reduced to hydroxylysine-6- C^{14} and this was converted to lysine 6- C^{14} . Rothstein & Claus (283) brominated ethyl- α -acetamido- α -carbethoxy- β -hydroxyvalerate. The resulting bromo compound was reacted with $KC^{14}N$. The nitrile was then either reduced to lysine or hydrolysed to α -aminoadipic acid. Arnstein *et al.* (284) prepared carboxyl-labeled lysine by an adaptation of Adamson's synthesis. These same workers prepared lysine labeled in the alpha-carbon through the condensation of 4-iodobutylphthalimide with ethyl phthalimido (α - C^{14}) malonate. They furthermore prepared N^{15} -labeled-L-lysine from ϵ -benzoyl-D-lysine by forming the ϵ -benzoyl-bromohexonic acid, then converting this into ϵ -benzoyl-L-lysine with N^{15} ammonia, a reaction that produces a Walden inversion.

A general biochemical synthesis for isotopically labeled amino acids is the reversal of enzymic decarboxylation. Hanke, Summaria & Mandeles (285) have thus prepared glutamic acid from γ -aminobutyrate and $C^{14}O_2$ in the presence of glutamic decarboxylase using Amberlite IR4-B resin as a trap to displace the equilibrium. Although decarboxylations appear to go to completion, there must of course be an equilibrium. The resin, being a weak base, adsorbs glutamate but not γ -aminobutyric acid or CO_2 and displaces the equilibrium in favor of carboxylation. There was a small, but significant, accumulation of C^{14} -labeled glutamic acid on the resin.

Biological synthesis of uniformly labeled L-amino acids is very attractive. The principal disadvantage for an investigator interested in certain specific problems is that only a small portion of the administered activity is incorporated into any single carbon, of any single amino acid. A good estimate of the potentialities of biological synthesis may be obtained from the papers of Schieler, McClure & Dunn (286) who employed *Chorella pyrenoidosa*, from Tarver *et al.* (287) who used *Rhodospirillum rubrum*, and from Frantz *et al.* (288) who used *Thiobacillus thioxydans*.

Since many syntheses require NaC^{14}N and NaCN^{15} , it should be noted that MacDiarmid & Hall (289) have described a simplified preparation of NaC^{14}N and NaCN^{15} by a modification of Adamson's method (heating barium carbonate and sodium azide).

Amino acid derivatives of carbohydrates.—Amino acid derivatives containing carbohydrate residues linked in various ways have been prepared. One type is represented by the acylglucosamines. Doherty, Popenoe & Link (290) have made N-(carbobenzoxy-L- α -glutamyl)- β -D-glucosamine and N-hippuryl- β -D-glucosamine by the reaction of acyl amino acid chlorides with 1,3,4,6-tetracetyl- β -D-glucosamine. N-glucosides constitute the second class of compounds. For example: 1-fluoro- β -glucose was reacted with the amino groups of glycine, serine, lysine, and sarcosine to form the corresponding N-glucosides [Micheel & Klemer (291)]. When anhydrous D-glucose and DL-phenylalanine were boiled in dried methanol, on the other hand, a N-substituted fructosamine, fructose-phenylalanine, was formed [Gottschalk (292)]. In a third type of derivative, a carbohydrate which contains a carboxyl group is reacted with the amino group of an amino acid to form a glyconyl peptide. Some 40 examples of this new class of compounds have been synthesized by Doherty (293). The free carboxyl groups of alginic acid have been combined with the amino groups of glycine, lysine, arginine, cystine, tyrosine, and glycylglycine [Micheel & Mille (294)].

The synthesis of some N-phosphoryl amino acid esters is reported by Li (295) while some acyl phosphates were made by Bentler & Netter (296).

Methods for the determination of amino acids.—Of the three categories, chromatographic, microbiological, or chemical, certainly the chromatographic procedures have received the most attention during the past year.

A very interesting comparison of analyses by three groups of investigators of the same sample of sheep adrenocorticotrophic protein is reported by Mendenhall (297) and several co-operating analysts. The results are tabulated for two microbiological methods and one chromatographic (Stein & Moore's starch column method). The greatest variation (2-fold) was observed in the analyses for methionine. Analyses for proline, tyrosine, arginine, and cystine also showed large deviations of the order of 20 per cent. The serine and threonine data are hard to interpret because the methods of correcting for loss during hydrolysis are not given. The data for the other amino acids showed a spread of 10 per cent or less. There is no evidence of a methodological error since as much variation occurred between results by the two microbiological assays as between these values and those obtained by the chromatographic technique.

Many very satisfactory methods for the paper chromatography of amino acids have appeared. For example, the procedures of Redfield (298) and Levy & Chung (299) for two-dimensional chromatography on paper are typical of sound paper chromatographic technique. Three new books have been published on chromatography which contain sections dealing with amino acids [Cramer (300); Samulson (301); Lederer & Lederer (302)]. A fourth by Brim-

ley & Barret (303) entitled *Practical Chromatography* is also recommended.

The determination of free amino acids in tissue filtrates presents several problems not encountered in determining the amino acid composition of protein. It is first necessary to remove the protein from the homogenized tissue or plasma. The precipitation of protein without appreciable loss of free amino acids has been studied in some detail by Van Slyke & Hiller (304), who found that picric acid, trichloroacetic acid, and tungstic acid were satisfactory precipitants but that ethanol was not. It is possible to remove picric acid, prior to chromatography, with Dowex-2 (305); trichloroacetic acid may be extracted with ether (193); and tungstate may be employed in such a manner that little excess reagent remains in the filtrate (307).

Once a filtrate is prepared it is almost always necessary to remove salts,—which interfere in chromatography. Modifications of the electro dialysis procedure of Consden, Gordon & Martin (308) are usually employed [Block & Bolling (309)].

Several methods have been proposed to circumvent electrolytic desalting. One is to employ ion exchange columns to absorb cations and anions under such conditions that amino acids can be eluted [Redfield (298); Carsten (310); Piez, Tooper & Fosdick (311)]. McCollum & Rider (312) suggest a chemical method in which the amino acids are extracted from salt by a solution of DL-camphorsulfonic acid in acetone. The dissolved amino acids are recovered by saturating with ammonia gas.

Another way of circumventing desalting would be to convert the amino acids into ether-soluble derivatives, which could then be chromatographed. Weibull's (313) method for the quantitative determination of dinitrophenyl amino acids on paper chromatograms might be suitable.

An example of the application of paper electrophoretic techniques to the determination of the free amino acids of serum may be found in the paper of Dubreuil, Auclair & Timiras. (314).

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FAT-SOLUBLE VITAMINS^{1,2}

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VITAMIN A AND CAROTENE

CHEMISTRY

Syntheses of vitamin A and of related compounds are reviewed by Baxter (1). Variants of vitamin A acid, including the *o*-tolyl and mesityl analogues (2) and a 4-carboxy compound (3) were made. The *o*-tolyl compound had no biological activity, and the other two compounds had only slight activity. A pentaene ester, related to vitamin A acid but possessing an unmethylated ring system, was synthesized. It proved to be highly unstable (4).

Farrer *et al.* (5) synthesized vitamin A₂ via 3'-dehydrovitamin A₁ acid. The absorption spectrum of the synthetic vitamin A₂ and that of its anti-mony trichloride reaction product agree well with those of natural vitamin A₂, reported by Shantz (6). Evidently, vitamin A₂ (See Fig. 1) is 3'-dehydrovitamin A₁ (5, 7). The biopotency of the synthetic (all-*trans*) preparation in rats was 30 per cent that of vitamin A₁, compared to 40 per cent reported for the natural compound (8).

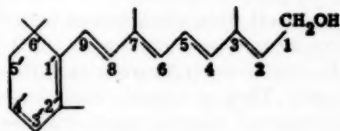


FIG. 1. Vitamin A₂.

Karrer & Kebrle (9) report that some unsaturated compounds, previously made from β -ionone, are not β -ionylidene derivatives but have the *retro*ionylidene³ structure. Meunier *et al.* postulated anhydro vitamin A to have a *retro* structure in 1943 (10). Subsequently, Shantz (11) proposed a *retro* structure for a naturally-occurring compound with some vitamin A potency, and Robeson (12) formulated a synthetic compound as a *retro* derivative. Oroshnik *et al.* afforded proof of structure of the *retro*ionylidene series by degradative studies (13). They showed that, as a rule, *retro* com-

¹ The literature survey for this chapter was ended in December, 1953.

² Communication No. 200 from Biochemistry Department of Distillation Products Industries.

³ The term *retro* is used to indicate the occurrence of a conjugated system one carbon atom back into the ring (13) with the resulting change from a β -ionone to an α -ionone ring.

pounds are preferentially formed on dehydration of a β -ionol or its vinylog (14).

Some spectroscopic properties of vitamin A hydrocarbon are given (15). The compound obtained by Oppenauer oxidation of vitamin A with diethyl ketone as hydrogen acceptor, which was previously believed to be dehydroretinene, is a C_{26} ketone [Hamlet *et al.* (16)].

The five crystalline retinenes which have been isolated seem to be *cis-trans* isomers of one another [Hubbard, Gregerman & Wald (17)]. If so, one of them must contain a "forbidden" *cis* linkage. This is no longer an objection, since stable compounds containing such hindered *cis* bonds continue to be made (18, 19). Collins *et al.* (20), using rat retinas and choroids, observed *in vitro* regeneration of rhodopsin in a medium containing synthetic vitamin A alcohol and phosphate buffer. The time required was 30 to 60 min., in good agreement with human dark-adaptation time. The known relations of the retinenes₁ and retinenes₂ to the biochemistry of vision are summarized by Wald (21). Wald *et al.* (22) have prepared a new visual pigment, designated as cyanopsin, by *in vitro* combination of retinene₂ and chicken cone opsin. It has maximum light absorption at 620 m μ . While it is presumed to exist in nature (e.g., in fresh-water fish), it has not yet been isolated.

The synthetic chemistry of the carotenoids is reviewed by Inhoffen & Siemer (23). The dimethyl ester of 8,8'-*cis*-crocetin was prepared by total synthesis. It was isomerized to the dimethyl ester of all-*trans*-crocetin, which is identical to naturally-occurring, so-called " γ -crocetin" (24). The direct total synthesis of all-*trans* methylbixin is described (25). Attempts to synthesize 3,4-3',4'-bisdehydro- β -carotene ("provitamin A₂") are recorded (26). Eugster *et al.* (18) obtained two *cis*- β -carotenes with *cis*-configuration at "hindered" double bonds. They are stable compounds which crystallize readily. *Cis*-lycopenes were synthesized by Garbers & Karrer (19), which also possess *cis*-configurations at "hindered" double bonds.

The reagent, N-bromosuccinimide, was used to produce carotenoid-like pigments from squalene (27), phytofluene and phytoene (28), and β -carotene (29). The latter reaction yielded three pure compounds, including dehydro- β -carotene (a *retro* compound) and a bisdehydro- β -carotene which has no provitamin A activity in the rat (29). Dehydro- β -carotene was converted, via its boron trifluoride complex, into an isomer of cryptoxanthin (30). Termed "isocryptoxanthin," the new compound is 4-hydroxy- β -carotene. It and its methyl ether have provitamin A activity in the rat equal to about one-half that of β -carotene (31). Spectral data on some stereoisomers of dehydro- β -carotene are given (32).

That α -tocopherol effectively inhibits hemin-catalyzed oxidation of unsaturated fat and concurrent oxidative destruction of vitamin A *in vitro* was shown (33, 34). Tocopherol protects against such destruction of vitamin A in oily medium (33) or in fat-water dispersion (33, 34). The multiple factors which affect carotene stability in alfalfa during preservation (35) and during storage (36) are pointed out.

ANALYSIS

The 1953 edition of the British Pharmacopoeia (37) now describes for vitamin A a physicochemical assay only. The biological assay is omitted. The procedure given is much like that in the Addendum to the previous edition (38), and it follows rather closely the recommendations of the Subcommittee on Fat-Soluble Vitamins, appointed by the World Health Organization (39).

Evidence accumulates that the specific absorbancy ($E_{1\text{cm}}^{1\%}$) of vitamin A alcohol in isopropanol is about 1,835 (40, 41, 42). This figure being about 5 per cent higher than the value specified in the U. S. Pharmacopoeia XIV (43), suggests that the U.S.P. XIV value may need to be revised.

Oils can be tested, spectrophotometrically, for the presence of both vitamins A₁ and A₂, according to Cama & Morton (44). One determines the $E_{1\text{cm}}^{1\%}$ at 326 μ , 351 μ , and 286 μ (on the unsaponifiable fraction) and at 693 μ and at 620 μ in the antimony trichloride color test. The 693 μ absorption measures vitamin A₂ directly, and from it the vitamin A₂ contributions to the ultraviolet absorption at 351 μ and 327 μ can be calculated. A conversion factor is given for calculating the probable vitamin A₂ contribution to the potency.

Murray & Campbell (45) compared results of physical and chemical methods for vitamin A assays with results of biological assays. Based on their findings, they recommended the Morton-Stubbs correction procedure applied to the nonsaponifiable fraction of oils (as in the U.S.P. XIV method), as the best method for routine estimation of vitamin A in fish liver oils and concentrates. They found the biopotency of neovitamin A to be 72 per cent of that of all-*trans*-vitamin A, when assayed by the vaginal smear method.

Tocopherols in large amounts interfere with the determination of vitamin A by the U.S.P. XIV method. The interference can be circumvented by destroying vitamin A in the mixture by means of sulfuric acid and comparing the absorbancies of the treated and untreated mixtures (46). Alternately, the vitamin A can be separated from α -tocopherol by chromatography on deactivated alumina (47) or on silicone-impregnated paper (48) prior to spectrophotometric measurement.

Reports on collaborative assays of vitamin A in margarine (49) and in mixed feeds (50) have appeared. Samples of alfalfa meal are stable with respect to carotene content during the time required to mail them to collaborators (51). The bioassay vitamin A value of an extract of yellow corn meal agreed well with the vitamin A potency calculated from chemical analysis, provided that hydrated lime was used as the adsorbent to separate an inactive pigment and partially-active isomers of β -carotene from the all-*trans*- β -carotene [Callison *et al.* (52)]. A possible source of error in the analysis of alfalfa meal for carotene content is the presence of stabilizers such as N,N'-diphenyl-*p*-phenylenediamine (53).

Procedures for the chemical assay of vitamin A and carotenoids in hens' eggs (54), of vitamin A in foods and feeds (55), and of vitamin A in feces

(56) are described. Satisfactory recoveries of added vitamin A are reported for these three methods. The method of choice for determining the vitamin A content of calf plasma seems to be the antimony trichloride method without either saponification or separation of carotenoids (57).

Alpha ionone gives a red color with alcoholic alkali, while β -ionone does not. The reaction can be used to determine α -ionone in the presence of the β -ionone [Karrer & Blass (58)]. Factors which affect the determination of vitamin A with inorganic halides are critically discussed (59). The compound, thymyl *p*-phenylazobenzoate, has a spectral absorption curve closely resembling that of vitamin A acetate (60). It might be useful as a standard reference substance in the spectrophotometric determination of vitamin A.

OCCURRENCE

Goodwin has written a book on the comparative biochemistry of the carotenoids (61) and a review on biogenesis of carotenoids (62).

Numerous reports concern the detection and isolation of carotenoids in plant material (63 to 69), in various microorganisms (70 to 76), in marine "red water" (77), in the crab (78), and in the fresh-water mussel (79). Clues to the pattern of carotenogenesis are provided in a number of papers (71 to 75, 80, 81, 82). Carotenoids were reviewed by Mackinney in the 1952 volume of this series (83), and these reports will not be considered in detail.

Phytoene and phytofluene, hitherto undetected in green plant tissue, have now been found in alfalfa (63) and in green leaves of *Hevea brasiliensis* (82). Spectral properties of all-*trans*-phytofluene and of a *cis*-phytofluene are given by Koe & Zechmeister (65).

Measured by the Robeson-Baxter method, neovitamin A constituted 11 to 39 per cent of total vitamin A in natural concentrates and fish liver oils (84). Carotenoid pigments of the crab (*Carcinus maenas*) include β -carotene, supplied by food, and esters of astaxanthin, formed by the organism from β -carotene, as well as intermediate oxidation products (78). The fresh-water mussel (*Anodonta cygnea*) resembles marine lamellibranchs in that it accumulates more xanthophylls than carotenes. Zeaxanthin is its main pigment. It does not synthesize new and characteristic xanthophylls (79) from ingested carotenoids. Ganguly *et al.* isolated and crystallized a pigment from sea urchins (*Strongylocentrotus purpurata* and *Strongylocentrotus franciscanus*), which is probably echinenone. Its provitamin A activity in the rat was 44 per cent that of all-*trans*- β -carotene (85).

Fisher, Kon & Thompson (86, 87) made extensive studies of the distribution of carotenoids in marine and Mediterranean Crustacea. Euphausiids from both sources had, in addition to large quantities of astaxanthin, high concentrations of vitamin A but no β -carotene. The eyes of euphausiids contained over 90 per cent of the vitamin A in the organism. In contrast to euphausiids, the decapods contained lower concentrations of vitamin A and total carotenoids, the difference being in the eyes. Astaxanthin was present in measurable amounts. The rich liver stores of vitamin A in whales is ac-

counted for by the high vitamin A content in euphausiids forming their food.

Collins, Love & Morton (88, 89, 90) studied distribution of vitamin A₁ and A₂ in several species of animals. One species, *Amblystoma tigrinum*, seems to require little or no vitamin A or else to be able to use an unrecognized compound other than vitamin A₁ in physiological processes (88). Lipids of two species of newt (*Triturus cristata* and *Triturus carnifex*) contain both vitamins A₁ and A₂, as well as carotenoids (89). Tadpoles of *Rana esculenta* and *Rana temporaria* have vitamin A₁ only (90). This contrasts with Wald's earlier report that tadpoles of *Rana catesbiana* contain vitamin A₂ but that after metamorphosis the adults contain vitamin A₁ only. Collins *et al.* (90) conclude that vitamin A₂ may result from (a) preformed vitamin A₂ in the diet, (b) the conversion of β -carotene and other provitamins to vitamin A₂, and (c) the conversion of a specific provitamin A₂, which might be an animal carotenoid such as astaxanthin. So far there is no direct evidence for this last suggestion.

Grimbleby & Black (91) suggest that the hen is able to alter other carotenoid pigments to cryptoxanthin, a vitamin A-active pigment. According to Ganguly, Mehl & Deuel (92), only traces of carotenes, but large amounts of cryptoxanthin or zeaxanthin, appear in blood and tissues of chickens fed these substances.

Animals fall into four groups insofar as their ability to absorb and deposit different carotenoids is concerned. These groups and examples of animal species belonging to them are: Group *a* (low carotene, low carotenol), rat and ewe; group *b* (low carotene, high carotenol), chicken; group *c* (high carotene, low carotenol), cow; group *d* (high carotene, high carotenol), frog. This divergent behavior may be attributable to specific protein receptors for vitamin A and for the carotenoids present in different tissues [Ganguly, Mehl & Deuel (93)].

PHYSIOLOGY

Requirements.—Almquist (94) estimates the minimum vitamin A requirement for growth of the chick is approximately 1,000 I.U. per lb. of diet, while that of the poult is approximately 2,300 I.U. per lb. of diet. Vitamin A intake at a level of 1 I.U. per day is more than sufficient to supply all nutritional needs of the growing and adult mouse, according to the experimental results of McCarthy & Cerecedo (95). The minimum safe level of carotene intake for successful reproduction in Guernsey cattle appears to be 90 μ g. per lb. of body weight daily (96).

Relation of intake to tissue levels.—Thomas & Moore (97) found a linear relation between the level of carotene intake and the extent of liver storage of both carotene and vitamin A for calves on a ration of natural feedstuffs. Ganguly & Krinsky (98) were unable to confirm a previous report of Glover *et al.* (99) to the effect that a direct relationship exists between vitamin A alcohol levels in blood plasma and liver of rats. In experiments of Booth (100), to be cited later, liver vitamin A varied directly with intake over a

wide dosage range in rats. According to Almquist (101), the blood plasma vitamin A level is linearly related to the logarithm of liver vitamin A content, as well as to the logarithm of dietary vitamin A intake. This general relationship holds for mammals (e.g., rats, lambs, and cattle), as well as fowl.

Conversion of provitamin A and absorption of vitamin A.—Confirmation that vitamin A is formed from provitamin A in the small intestine is provided in recent studies. Bernhard (102) found that equally as much vitamin A was recovered from intestinal lymph as from thoracic-duct lymph (which contains liver and intestinal lymph) following oral dosage of rats with β -carotene. Recovery of vitamin A in thoracic-duct lymph following oral administration of vitamin A, β -carotene, or α -carotene, was about 40 per cent, 6 per cent, and 4 per cent, respectively. Traces of unchanged carotenes appeared in the lymph (102, 103).

Rosenberg & Sobel (104) used a method of analysis, which they consider specific for vitamin A (difference spectrum), to demonstrate conversion of carotene to vitamin A in the isolated small intestine of the rat. However, the validity of this procedure is questioned by Bieri & Pollard (105); in similar experiments they used other analytical methods for vitamin A and obtained negative results. Alloxan-diabetic rats stored only one-fourth as much vitamin A in the liver after a fixed dose of carotene as did nondiabetic controls (106).

Bieri & Pollard (107) claim that formation of vitamin A from carotene by the rat can occur in tissues other than the small intestine. Church *et al.* (108) aver that there is a major difference in the manner in which cattle and sheep utilize intravenously-injected carotene. Presumably, sheep can convert it into vitamin A, while cattle cannot, to any appreciable extent. Follow-up of these observations should provide very interesting information.

Giving cortisone in large doses seems to impair the conversion of carotene to vitamin A in the rat (109). Morgan & Arnrich (110), studying carotene utilization in rats and dogs given thiouracil, concluded that a normally functioning thyroid is not necessary for carotene utilization. Addition of 0.5 or 1.0 mg. of α -tocopherol daily to the diet of rats receiving generous supplements of vitamin A or of carotene failed consistently to cause increased liver storage of vitamin A (111).

Young calves received colostrum and then early milk from birth to 17 days of age. During this period the average apparent absorption of vitamin A was 81 to 95 per cent, while that of carotenoids was only 38 to 65 per cent (112).

Gounelle *et al.* (113) and Katsampes *et al.* (114) showed that vitamin A aldehyde (retinene) is readily absorbed and converted into vitamin A by normal humans. It is therefore a good source of vitamin A. That retinene₁, fed to rats, is reduced in the lining of the intestine to vitamin A₁ and then esterified and carried to the liver and stored was previously shown (115). Similarly, retinene₂ is converted to vitamin A₂ by the rat and stored as such (116).

Transport and deposition in body tissues and fluids.—Ganguly & Krinsky (98) determined the rate of appearance of vitamin A ester and alcohol in plasma and liver of rats given a single dose (14 mg.) of vitamin A ester. In plasma the ester form showed a typical sharp peak at 5 to 6 hr., and the free form showed relatively less increase. In liver the ester form continued to be deposited up to 18 hr. after dosing, whereas the alcohol form increased for only 5 hr. and was then maintained at a fairly constant level.

High & Day (117) determined vitamin A present in livers, kidneys, and plasma at intervals after feeding a single oral dose (0.1 mg.) of vitamin A to A-depleted rats. A plasma level of 18 to 30 $\mu\text{g.}$ of vitamin A per 100 ml. was reached before liver storage occurred. Vitamin A first appeared in the blood, then the liver, and finally the kidneys.

Booth (100) describes some interesting experiments concerning the sex difference in liver storage of vitamin A. Female rats, given carotene in oil or as vegetables, consistently stored more vitamin A than did males. Both lost their stores at similar rates; hence livers of males depleted sooner than those of females. Both sexes had similar kidney levels shortly after dosage with vitamin A. The level in kidneys of male rats increased with time, but in kidneys of females no comparable rise was found. Rats received doses of vitamin A for nine days, at graded levels, and were killed two days later. Curves of liver vitamin A versus dose were linear over a 60-fold range for the two sexes, but the slopes were different. The difference in liver storage between males and females was, therefore, not a constant one but was proportional to dose. Although the explanation of the sex difference in liver storage is not known, it is apparently not related to growth rate.

The length of survival of adult bobwhite quail which were starved was not related to vitamin A content of their livers [Harper *et al.* (118)]. Rowland *et al.* (119) found that in the cow the daily concentration of vitamin A and carotenoids during the period of prepartum milking was negatively correlated with the daily weight of fat and positively correlated with the daily weight of globulin produced. Carotene intake at 60 $\mu\text{g.}$ per lb. body weight per day was inadequate, and intake at 333 $\mu\text{g.}$ was more than adequate, to maintain liver stores or plasma vitamin A levels of beef cows during the last six months of gestation [Baker *et al.* (120)].

Chanda (121) determined partition of carotenoids and vitamin A in the colostrum and milk of cows and goats as affected by diet, parity, and stage of lactation. Goat colostrum contained β -carotene, while mature goat milk did not. Cow colostrum and milk and goat colostrum contained some vitamin A alcohol, but none was present in goat milk. A parabolic relationship between the stages of lactation and the vitamin A content of milk fat was found in both species.

Somewhat conflicting are the reports on intracellular distribution of vitamin A and of vitamin A esterase activity (122 to 125). It would seem that further work is needed to clarify this.

Effect on bone.—Vitamin A deficiency in young ducks causes marked re-

tardation and suppression of endochondral bone growth (126), while excess vitamin A accelerates these changes (127). Alkaline phosphatase content of the epiphyseal junction of the bone is reduced in a state of vitamin A deficiency and increased in hypervitaminosis A in rats [Ludwig (128)].

Metabolic interrelationships.—Weanling rats were thoroughly depleted of vitamin A with the use of exhaustively-extracted casein in the diet. A molecular distillate of lard, which was apparently free of vitamin or provitamin A, given at the level of 0.6 gm. per day, restored growth and cured xerophthalmia in these rats [Lowe & Morton (129)]. This finding, confirming an earlier report of Kaunitz & Slanetz (130), is considered by Lowe & Morton to be indicative of the presence of a vitamin A-replacing factor in lard. On the other hand, Herb *et al.* (131) were able to isolate fractions from lard, which gave positive Carr-Price tests and a typical vitamin A spectral curve. They conclude that the biological vitamin A activity of lard is largely attributable to the actual presence of vitamin A.

Interesting new effects of a state of extreme vitamin A deficiency in the rat are reported by Lowe, Morton & Harrison (132). Notable changes in the histochemical appearance of the adrenal cortex occurred. Abnormal metabolites appeared in the liver lipid, characterized by $\lambda_{\max.}$ at 275 $m\mu$. Lowe *et al.* believe that these metabolites cannot arise from vitamin A and that they may result from a lifting of restraint on the dehydrogenation of cholesterol or steroid hormones.

Hypervitaminosis A was produced in guinea pigs, but no clear evidence was obtained that increased intake of vitamin C had an ameliorative effect or that a disturbance of ascorbic acid metabolism had been induced (133). Feeding lecithin in addition to vitamin A did not increase plasma vitamin A levels of calves over those of controls, and it did not consistently increase their liver stores in experiments of Aschaffenburg *et al.* (134). Neither aureomycin (135) nor vitamin B₁₂ (136) appears to affect the utilization of pre-formed vitamin A in rats; but vitamin B₁₂ did increase tissue deposition of vitamin A when carotene was fed (136).

Ross & Gallup (137) previously observed an inverse relationship between blood plasma inorganic phosphorus and plasma carotene levels in beef cattle fed phosphorus-deficient rations. Klosterman *et al.* (138) now report that in rats, given equal amounts of carotene or vitamin A, the liver stores of vitamin A are inversely related to the phosphorus content of the rations.

A liberal allowance of vitamin A enabled young rats at least partially to resist the ill effects (e.g., weight loss) of a severe deficiency of protein (139). Comparisons were made of some effects of casein, lactalbumin, gluten, and zein on the nutritional utilization of β -carotene by growing albino rats (140). Rats receiving casein stored two to five times as much vitamin A in their livers as did the other rats, even though the livers of casein-fed rats were one-fifth smaller. The authors suggest, therefore, that, "Vitamin A is consumed in a liver process which transforms moieties from the ingested protein to other proteins or amino acids."

Effect of extraneous factors on vitamin A metabolism.—Blood plasma vitamin A levels increased 14 to 18 $\mu\text{g.}$ per 100 ml. within 4 hr. after ethanol was administered to calves and goats that were on adequate vitamin A rations. Virtually no increase occurred in vitamin A-depleted animals given ethanol. Subsequently, livers of these animals were assayed for vitamin A. There is a direct relationship between the amount of vitamin A stored in the liver and the amount of increase in plasma vitamin A levels following ingestion of alcohol (141). It is suggested that the phenomenon might be used as a means of evaluating the true vitamin A nutritional status of large animals. Perhaps it could be applied to humans also.

Exposure to x-irradiation (500 to 650 r) did not affect liver vitamin A stores of rats (142). Growth of rats fed a diet deficient in vitamin A was unaffected by conditions of extremely low or extremely high humidity (143). Apparently, mastitis in cows causes enhanced leakage of blood plasma lipids into the milk through the damaged glandular epithelium. Milk of infected cows contains more carotenoids and vitamin A alcohol in the milk fat than does milk of uninfected cows (144). Fowl, infected with intestinal coccidiosis, had liver vitamin A reserves less than one-tenth those of control, uninfected birds. Both groups received a balanced ration with adequate vitamin A as carotene (145).

Miscellaneous.—It appears that high vitamin A intake in the form of synthetic vitamin A palmitate in margarine is not harmful in the rat (146).

Explants of chick ectoderm, grown in a tissue culture medium containing excess vitamin A, failed to keratinize. Instead, the cells were differentiated into mucus-secreting, often ciliated epithelial cells, resembling those of normal nasal mucosa (147).

Recent surveys of blood vitamin A and carotene levels in groups of human subjects were made in the Philippines (148), Great Britain (149), and Scotland (150).

VITAMIN E

HUMAN NUTRITION

The role of vitamin E⁴ in human nutrition is not yet defined. But in recent years chemical analysis for vitamin E has provided much information about the distribution of vitamin E in human tissues and its physiological variations. A brief account of pertinent facts and of recent contributions to the subject will be given. Papers on tocopherol in human disease are not considered here.

Blood serum tocopherol levels of normal human adults in the United States ordinarily range from about 0.5 to 1.5 mg. per 100 ml. (151). A group of nutritionally-deficient patients had lower blood tocopherol levels than did

⁴ Vitamin E as used here means α -tocopherol and certain of its esters. The non- α -tocopherols, in the opinion of this reviewer, can be disregarded as sources of vitamin E-active material for animals, including humans.

healthy control subjects from the same geographical area (152). In England, blood plasma tocopherols of healthy private patients averaged 1.31 mg. per 100 ml. and of hospitalized mental patients 1.15 mg. per 100 ml. There was little seasonal variation (153).

There is no constant pattern of variation in blood vitamin E content in women during the menstrual cycle (151). The blood tocopherol level increases during pregnancy and is about two-thirds higher at term than in the non-pregnant woman (154).

Cord blood of newborn infants has about one-fifth the tocopherol content of the mother's blood (154). The average level of vitamin E in cord blood equals the average level of the infant's blood taken within the first 24 hr. (155). The level is apparently independent of sex or birth-weight (154, 155).

Red cells of newborn infants could be hemolyzed by hydrogen peroxide *in vitro*; even though the mother received high daily doses of vitamin E during the last weeks of pregnancy [György *et al.* (156)]. On the other hand, the mean tocopherol level in the cord blood of newborn infants was significantly higher when the mother received large α -tocopherol supplements during labor than when not given a supplement [Minkowski *et al.* (157)]. Apparently, significant but limited amounts of α -tocopherol traverse the placental barrier in humans.

That the relatively low blood tocopherol levels found in human newborns (154, 155, 158) reflect low tissue tocopherol stores is shown by analytical results of Dju, Mason & Filer (159). They found that tocopherol concentration is low in the entire fetus and in individual tissues and organs during the second to the sixth month of gestation. At birth, either premature or full-term, tissue tocopherol levels are slightly higher. One full-term infant contained a total of 20 mg. of tocopherol. Neonatal tissues average about 0.3 to 0.6 mg. tocopherol per 100 gm., compared to a probable value of the order of 3 mg. per 100 gm. for adult man.

Judged from the magnitude of increase in blood tocopherol level following an oral dose of α -tocopherol, premature infants can absorb vitamin E as well as full-term infants. This is in marked contrast to the diminished ability of the premature infants to utilize vitamin A and fats. Free and esterified tocopherol are absorbed equally well (160).

Cows' milk is a poor source of vitamin E. Mature human milk contains 2 to 4 times and human colostrum up to 20 times as much α -tocopherol as cows' milk (161, 162). Breast-fed babies show rapid increase in blood serum tocopherol content. Infants fed with cows' milk show little or no increase in blood vitamin E, and babies given partially-skimmed cows' milk formulas have an actual decline in blood tocopherol content [Wright *et al.* (158)].

No definite clinical significance is presently attached to the low vitamin E reserves of the human at birth and in early infancy. Susceptibility of red blood cells of infants to *in vitro* hemolysis by hydrogen peroxide was noted (156, 163). This can be reversed by giving α -tocopherol to the infant. The physiological significance of the test remains to be established.

However, the fact that the young animal of diverse species as rat, rabbit, lamb, calf, chick, and many others is especially prone to develop incapacitating symptoms of vitamin E deficiency, primarily muscle dystrophy or damage to the vascular or central nervous systems, coupled with the fact that many infants have low neonatal and postnatal tocopherol reserves, suggests that α -tocopherol may need to be added to the diet of the human infant.

Most American foods contain some vitamin E, but few foods have appreciable amounts of it (164). Vegetable oils, such as soy, cottonseed, wheat germ, and corn oils, are the richest sources of vitamin E in the diet. On the average, about 14 mg. of α -tocopherol are consumed in the diet per capita daily in the United States.

Tocopherol is not normally excreted in the urine. If present in human sebum, tocopherol does not exceed 0.03 per cent in amount (165, 166). Fecal excretion of tocopherol in a group of normal healthy adults averaged 21.6 mg. per day; this was 64.4 per cent of the calculated dietary intake. No tocopherol esters were found in the feces of individuals given large doses of α -tocopheryl acetate (167).

All tissues of normal human adults examined contained some tocopherol, predominantly α -tocopherol. Based on fat content, the tocopherol contents of various tissues of the same subject were similar. The normal adult has vitamin E reserves of the order of several grams [Quaife & Dju (168)].

Mason & Dju (169) measured the tocopherol contents of tissues of a score or more of human subjects of all ages. Based on unit weight of tissue, tocopherol values increase in childhood somewhat over levels at birth. Beyond childhood, most tissue tocopherol levels vary little and approximate the range of plasma tocopherol levels. An exception is adipose tissue in which tocopherols increase appreciably with age up to middle life and then tend to decrease.

Studies made by Quaife *et al.* show there is a fundamental relationship between intake of α -tocopherol and tissue deposition. In the rat, over a very wide dosage range, the logarithm of liver tocopherol content varies directly as the logarithm of ingested d - α -tocopherol (170). The tocopherol content of hens' eggs varies as the logarithm of ingested tocopherol [Dju, Quaife & Harris (171)], as does the tocopherol content of cows' milk [Swanson & Harris (172)]. In the rat, the tocopherol content of blood serum varied with the logarithm of d - α -tocopherol intake [Quaife (173)].

Apparently, the tocopherol content of blood serum and of certain other body tissues varies directly as the logarithm of liver tocopherol content and as the logarithm of ingested α -tocopherol. It seems probable that this relationship holds true for humans also.

FARM ANIMALS

The vitamin E nutrition of farm animals is critically reviewed by Blaxter & Brown (174).

Cattle, sheep, and swine.—The outstanding defect in vitamin E deficiency in farm mammals appears to be a hyaline degeneration of the skeletal and

cardiac musculature. Under some conditions, there may be abnormalities of fatty depots as well (174). Electrocardiograms of vitamin E-deficient lambs were abnormal. At autopsy, the lambs showed extensive necrosis of the heart muscle (175). Vitamin E deficiency affects beef calves with typical symptoms of muscular dystrophy. Lesions occur in the heart, diaphragm, tongue, and bilateral muscle groups. Some cases respond to 1,000 mg. α -tocopherol, orally, twice a day (176). According to Schofield (177), in addition to vitamin E deficiency, phosphorus deficiency may be a factor in the etiology of muscular dystrophy in the lamb and calf. Variation in vitamin E reserves of young calves is not the sole reason for variation in the incidence of muscular dystrophy (178). Addition of one to four ounces of cod-liver oil per day to a dried skim milk ration, fed to calves, caused death or severe muscle dystrophy in many of the animals. Replacement of the cod-liver oil with vitamins A or D prevented most of the dystrophy, and the further addition of α -tocopheryl acetate to the diet gave complete protection (179).

Gorton & Naftalin (180) induced massive edema (exudative diathesis) in pigs by giving them a diet rich in cod-liver oil. There was edema of the skin and of certain viscera. The liver was normal. The heart muscle had areas of hyaline necrosis. Fats from back tissues had high iodine and peroxide values and contained pigment similar to that described in avitaminosis E in the rat and chick.

Obel studied toxic liver dystrophy of swine (181). The naturally-occurring disease usually runs a short course, ending in sudden death. Clinical symptoms are not striking. They include dyspnea and digestive disturbances and paralysis or muscular weakness. Besides the liver pathology, stomach ulcers, "yellow fat," and waxy degeneration of the skeletal muscles and of the myocardium sometimes occur. The disease could be produced experimentally in young pigs with low vitamin E reserves by feeding a synthetic diet. The diet was vitamin E-free, low in protein and sulfur-containing amino acids, and high in unsaturated fat. Liver pathology resembled that of dietetic liver injury in rats, for which vitamin E and sulfur-containing amino acids have a protective effect. In the swine disease α -tocopherol, alone, was not protective. If lard were substituted for the cod-liver oil of the diet and α -tocopherol added, liver injury could be entirely prevented. Cystine or methionine supplements were also protective. Factor 3, discovered by Schwarz, was not studied in this disorder. Obel believes that toxins resorbed from the intestine are the immediate cause of the liver injury.

The variation in vitamin E content of cows' milk appears to depend more on the stage of the reproductive cycle than on the breed, age, season of the year, or feed (182). The tissue tocopherol reserves of a calf at birth totaled 120 mg., and its early postnatal tocopherol intake, if it received, partially or wholly, colostrum from its dam, would have been 55 to 103 mg. (178).

Poultry.—Young chicks develop nutritional encephalomalacia or "crazy chick disease" when fed certain diets deficient in vitamin E. They show incoordinated movements and gait and sometimes become prostrated. These effects are a result of lesions of the brain and central nervous system. Chick

encephalomalacia has occurred spontaneously in many areas of the United States in recent years. The exact cause of the disorder is not known (183).

Singsen *et al.* (184) systematically varied certain nutrients in the ration of the breeding hen and of the young chick in studying the origin of encephalomalacia. Chicks, hatched from vitamin E-deficient breeding hens and fed a vitamin E-low diet with 2 per cent fish oil, averaged about 50 per cent mortality from encephalomalacia. Addition of 4 to 8 I.U. of vitamin E per lb. of chick ration (which already contained 5 I.U. per lb.) prevented encephalomalacia. The chick vitamin E "requirement," therefore, is 9 to 13 I.U. per lb. of ration. But this requirement varies with the presence of "stress factors" in the diet, such as fish oil, and with protective factors, such as antioxidants and certain of the B vitamins.

A widespread disorder of young chicks, occurring in New Zealand in 1950 and 1951, was most likely of nutritional origin and could be prevented by α -tocopherol administration [Thompson & Smith (185)]. Clinical and pathological features closely resembled those of exudative diathesis, as described by Dam & Glavind in 1939. Fish oil in the diet did not exceed 1 to 3 per cent, so it was probably not the causative agent; "heated" grain may have been the precipitating factor, according to Thompson & Smith.

α -Tocopheryl acetate or cystine were protective, and methylene blue, *bis* (diethylthiocarbamyl) disulfide (Antabuse), and nordihydroguaiaretic acid were ineffective in preventing muscular degeneration in chicks reared on vitamin E-deficient, low-fat diets (186).

Under the experimental conditions used, turkey poults which receive practical-type rations, containing cod-liver oil, develop an enlarged hock disorder and fail to grow normally. The combined addition of 20 mg. of nicotinic acid and 5 mg. of α -tocopheryl acetate per lb. of diet completely prevents the disorder. Nicotinic acid and vitamin E together are effective in preventing the enlarged hock disorder of 8 to 20-week-old turkeys as well as that of young poults [Scott (187)].

Jensen (188) presents evidence that, "Vitamin E may be a more critical nutrient in turkey hen rations than previously considered." A marked depression in hatchability of eggs resulted when turkeys fed a practical-type diet were not supplemented with α -tocopheryl acetate.

Feeding vitamin E to chickens resulted in the deposition of a more stable skin fat (189). The tocopherol content of the fat is the principal, if not the only, fat component causing chicken fat to be much more stable than turkey fat (190). Adding α -tocopheryl acetate to the ration caused increased fat stability in both chicken and turkey (190).

OCCURRENCE IN FOODS AND FEEDS

The occurrence of α -tocopherol in cod-liver oil (191) and of δ -tocopherol in seaweed (192) was shown. The α - and γ -tocopherol contents of 16 varieties of crude peanut oil were 0.021 to 0.030 and 0.015 to 0.023 per cent, respectively (193).

The kinds and amounts of individual tocopherols present in common live-

stock feeds of the British Isles were determined (194). Grass, clover, and lucerne had 10 to 40 mg. of α -tocopherol per 100 gm. of dry matter. The tocopherol content of the grasses decreased as the grass matured. Artificial drying of grasses and hay-making caused significant losses of tocopherol. Root crops contained relatively little tocopherol. Appreciable amounts of β -tocopherol were found in barley, oats, and wheat. In maize, beans, and linseed most of the tocopherol present is the γ -form.

After treatment of flour with chlorine dioxide under commercial conditions, the tocopherol content was reduced about 70 per cent, compared with untreated flour. Since flour is a staple article of diet and since chlorine dioxide has replaced agene as the more commonly-used flour improver in the United States, the available vitamin E in the diet may be significantly decreased through its use (195).

ASSAY METHODS AND CHEMISTRY

The tenth edition of the National Formulary will include monographs on five vitamin E preparations (196). Methods of analysis are given. Pure α -tocopherol is titrated directly with ceric sulfate, and α -tocopheryl acetate is titrated following acid hydrolysis to the free form. In mixed tocopherol preparations the total tocopherol content is determined spectrophotometrically by the Emmerie-Engel reaction. Non- α -tocopherols are measured by the nitrosation technique of Quaife, and α -tocopherol is determined by difference. For labeling in terms of International Units of vitamin E the following equivalencies are listed: 1 mg. of *dl*- α -tocopheryl acetate equals 1 I.U.; 1 mg. of *d*- α -tocopherol equals 1.49 I.U.; 1 mg. of *d*- α -tocopheryl acetate equals 1.36 I.U.; and 1 mg. of *dl*- α -tocopherol equals 1.1 I.U.

Analysis of individual tocopherols in a mixture is facilitated by some new methods which employ polarography (197) or paper chromatography (198, 199, 200). A colorimetric method for the analysis of vitamin E in blood serum is given (201).

Inglett (202) obtained chemical evidence for the existence of transitory α -tocopheryloxy free radicals. They were formed from α -tocopherol by reaction with other free radicals, such as triphenylmethyl or benzoyloxy radicals.

METABOLIC EFFECTS

Muscle.—Patients with muscular dystrophy have tissue tocopherol stores comparable to those of normal persons. The possibility remains that there is a metabolic defect in the utilization of tocopherol in muscular dystrophy [Mason *et al.* (203)]. Orr & Minot (204) showed that patients with muscular dystrophy, but not those with progressive neuropathic atrophy or myasthenia gravis, excrete D-ribose (as a complex) in the urine. Quite plausibly, they suggest that this excretion is a result of a specific metabolic derangement in muscular dystrophy and not simply a result of muscular wasting. Muscle slices from vitamin E-deficient hamsters showed decreased rate of release of amino nitrogen, possibly attributable to decreased rate of protein synthesis

[Milman *et al.* (205)]. Dystrophic muscle from calves had decreased potassium, creatine, and globulin nitrogen content and increased sodium, water, fat, and collagen nitrogen content, compared to normal muscle [Blaxter & Wood (206)]. Injection of rats with α -tocopheryl phosphate suppresses glycogenolysis in skeletal muscle (207). This effect is apparently exerted at the level of the phosphoglucomutase system (208, 209).

Although α -tocopherylhydroquinone disuccinate disodium salt has anti-dystrophic activity in the rabbit, it does not prevent resorption gestation in the pregnant, vitamin E-deficient rat. Evidence was also obtained that α -tocopherylhydroquinone is not converted to α -tocopherol to a significant extent in the body (210).

Liver necrosis.—Rats, which survived on a necrogenic diet, had a higher urinary excretion of ether-soluble acids than did rats on a similar diet treated with penicillin or aureomycin. However, vitamin E supplementation did not affect the excretion rate. Methyl malonic acid formed a large part of the total ether-soluble acids excreted by the untreated and vitamin E-treated animals; it formed a small fraction of the acid excretion of animals receiving penicillin or aureomycin (211). The content of coenzyme A in the livers of necrotic rats varied inversely with the degree of hepatic necrosis (212). The inclusion of α -tocopherol in the diet had no detectable effect on biochemical changes in the liver produced by a necrogenic diet, namely a fall in the levels of reduced glutathione and ascorbic acid (213). The levels of these two constituents, as well as those of nonprotein and total nitrogen, water, and glycogen, were similar in livers made necrotic by dietary means or by acute circulatory congestion (214). Hepatic necrosis and fibrosis could be produced in rats simply by injecting water into the portal vein (215). Little or no protection against liver necrosis in rats was found when γ - and δ -tocopherol were fed on a molar basis equivalent to that at which α -tocopherol gave protection (216).

Effect of highly unsaturated fat.—The greater the degree of unsaturation of dietary fat, the higher is the content of hepatic ceroid in rats fed a diet deficient in choline and somewhat low in vitamin E (217). Ceroid-like pigments occur in atheromatous lesions of the coronary, cerebral, renal, iliac, and femoral arteries, as well as the aorta, in man (218). Lipoperoxides occur in lipid of atherosclerotic aortas but not in normal aortas of man. The degree of atherosclerotic lesions (calcification plus atheromatosis) correlates well with the peroxide value (219).

Such findings indicate that abnormal fat oxidation in man, as in other animals, may initiate or accompany pathological processes. Because of the well-known role of α -tocopherol in preventing deposition of abnormal pigments or fat peroxidation in experimental animals, it would seem worth-while to study this interrelationship.

Vitamin E and physiological antioxidants.—The previous observations of Dam *et al.*, concerning the protection afforded by certain redox substances against some effects of vitamin E deficiency, have been extended. Methylene blue improved markedly, as did vitamin E, the reproductive capacity of fe-

male rats, reared on vitamin E-deficient, "fat-free" and cod-liver oil-containing diets [Dam & Granados (220)]. Moore *et al.*, on the other hand, found methylene blue ineffective in preventing resorption gestations in rats depleted of vitamin E. It did prevent brown discoloration of the uterus and degeneration of the kidney tubules of rats (221).

Methylene blue or α -tocopherol supplements decreased the amount of hepatic ceroid otherwise formed in rats fed a hypolipotropic, vitamin E-deficient diet (222). Methylene blue and, to a lesser degree, yeast nucleic acid, as well as α -tocopheryl acetate, protected rats against liver damage and mortality occasioned by giving them pyridine (223). Ingestion of α -tocopherol or of methylene blue prevented muscular dystrophy in calves fed a vitamin E-deficient diet with cod-liver oil. Calves given methylene blue had no more tocopherol in blood serum, muscle, liver, or perinephric fat than did control calves which succumbed to dystrophy (224).

The explanation of how such redox substances as methylene blue can protect against these effects of vitamin E deficiency is not known. Do these antioxidants "spare" tocopherol in the intestinal tract or in the body tissues? Do they protect some enzyme system, normally protected by vitamin E? Do they fulfill the antioxidant function but not the primary "vitamin" role of α -tocopherol? There seems to be no unequivocal answer to these questions, as yet.

Miscellaneous.—There was no difference in the arginase activity of livers and kidneys of normal, vitamin E-deficient, and arginine treated, vitamin E-deficient rats (225). Vitamin E-deficient rabbits had increased xanthine oxidase activity in the liver (226). The increase in xanthine oxidase occurred in the presence or absence of added dietary molybdenum; the molybdenum content of various rabbit tissues was not altered by the presence or absence of vitamin E in the diet (227). Injection of dogs with alloxan caused increased zinc content of blood serum and urine. These changes were counteracted by α -tocopherol, which was given to prevent hemolysis (228). A diet (semi-synthetic, containing 1 per cent wheat germ oil), which is adequate for full-term gestation in the *A strain* female mouse, is inadequate in this respect for the *Z strain* mouse. Fetal resorption in the *Z* mouse on this diet is prevented by omitting the trace minerals (cobalt, copper, zinc, manganese) or by adding α -tocopherol or vitamin B₁₂ to the diet (229). Giving tri-*o*-cresyl phosphate, a compound with so-called antivitamin E activity, to chicks inhibited the absorption of α -tocopherol or α -tocopheryl acetate from the intestinal tract (230).

ESSENTIAL FATTY ACIDS

Occurrence.—Chang & Watts have determined the content of essential fatty acids of a variety of cuts of meat before and after cooking (231). Chicken fried in vegetable oil and bacon as eaten are highest, and lamb and beef the lowest in linoleic acid content among the meats. Linolenic and arachi-

donic acid contents of the meats are small in comparison with that of linoleic acid. Ordinary cooking procedures cause little loss of these acids. Nor does baking or deep fat frying for several hours cause extensive loss [Chang *et al.* (232)]. The linoleic acid and total tocopherol contents of 22 animal and vegetable oils are correlated with a high degree of significance; $r = +0.79$ [Hove & Harris (233)].

The blood serum levels for two, three, and four double-bond fatty acids were determined for healthy children, who received a diet containing 3.4 per cent of total calories as linoleic acid. Expressed as per cent of total fatty acids in serum, mean values were: Dienoic acid 30.9, trienoic acid 2.0, and tetraenoic acid 12.7 [Wiese *et al.* (234)]. A semi-micro procedure for determining these constituents in blood serum, by spectral analysis of the alkali conjugated acids, is described by Wiese & Hansen (235).

Mechanisms of oxidation.—Further contributions to knowledge of the mechanism of autoxidation of linoleate (236), as well as to its lipoxidase-catalyzed oxidation (237, 238, 239), have been made. Tappel, Boyer & Lundberg (237) have proposed a reaction mechanism for the latter which involves formation of a biradical from linoleate and oxygen on the enzyme surface. The biradical may accept electrons from antioxidants or may react to give conjugated linoleate peroxide. Thus, linoleate could be considered as a prosthetic group of lipoxidase in its oxidation of secondary substrates. Holman has reviewed the arguments for and against the chain reaction nature of lipoxidase oxidation (240). Tappel was unable to find any significant quantities of lipoxidase in pork tissues (241). Lipoxidase is absent from the tissues of the rat, turkey, cattle, chicken, and various fishes (242). Tappel's findings reinforce the view that "lipoxidase activity" of animal tissues is actually attributable to the hemoglobin and myoglobin present. Tappel has reported on the kinetics of oxidation of unsaturated fatty acids catalyzed by hematin compounds (243).

Bioassay.—Thomasson has shortened the time needed for biological assay of essential fatty acids (244). The water intake of rats in the curative growth method of Deuel *et al.* (245) is restricted and standardized; this widens the response range considerably. A number of fatty acids were tested. All active ones had double bonds at the 6:7 and 9:10 positions, numbering from the terminal methyl group. Thomasson believes this structure to be requisite to vitamin F-activity. He determined potency of a large number of natural oils and fats by means of the biological method and also spectrophotometrically. Results show that vitamin F-activity is correlated mainly with conjugatable diene content, as determined spectrophotometrically (chiefly linoleic acid), and hardly at all with conjugatable triene (linolenic acid).

Deficiency effects.—Newly recognized consequences of lack of essential fatty acids in the diet include excess secretion of cerumen by the hamster (246), decreased capillary resistance and increased capillary permeability in the immature rat (247), and decreased rate of hair growth in the rat (248).

Lipolytic activity of the liver is markedly decreased; it can be restored to normal by adding arachidonic acid (0.5 gm. per day) to the ration and increased somewhat by a similar supplement of linoleic plus linolenic acid (249).

The skin lesion of the human disorder, phrynodema, is histologically like that of essential fatty acid deficiency in the rat and unlike those produced in the same animal by deficiencies of vitamin A or pyridoxine (250). Fat-deficient female rats showed, in addition to classical symptoms, a relative enlargement of the liver, heart, and kidneys, accompanied by large intertubular accumulations of blood [Panos & Finerty (251)]. Oestrus cycles were irregular and large numbers of "wheel cells" were present in the interstitial tissue of the ovary. A decrease in the number of acidophile cells in the pituitary occurred. The authors believe that fat deficiency causes a reduced secretion of luteinizing hormone by the pituitary with consequent derangement of ovarian function. Deuel *et al.* (252) depleted male and female rats of essential fatty acids and measured their survival time following exposure to x-irradiation. Survival time was significantly increased for those rats which received 10 mg. per day of methyl linoleate over that of rats receiving the basal, fat-free diet without linoleate.

Hansen, Wiese & Holmes (253 to 256) present results of long-term studies with young dogs on the need for fat in the diet. On a basal diet (1 per cent fat) dogs show dryness and desquamation of the skin, loss of hair, and increased susceptibility to infection. Complete cures were not observed at a fat intake of less than 16 per cent of calories. When 1 per cent of the calories was furnished by linoleic or arachidonic esters, definite improvement, but incomplete cure, occurred. The iodine numbers of serum fatty acids of the dogs, fed diets with and without fat, correlated well with the gross and microscopic appearance of their skin.

Metabolic interrelationships.—A number of recent papers discuss the relationship of essential fatty acids with other nutrients. Increasing levels of protein intake increased the arachidonate but not the linoleate or oleate percentages of lipids of liver, carcass, and brain in the rat; α -tocopherol had a variable effect [Hove & Hove (257)]. Higher carcass levels of linoleic acid and lower levels of oleic acid in the rat were associated with either cottonseed meal or soybean oil meal as the dietary protein than with fibrin, soybean protein and methionine, egg albumin, or casein [Tove *et al.* (258)]. Addition of 2 per cent cholesterol to a diet deficient in essential fatty acids caused deficiency symptoms to appear more quickly and with greater severity in the weanling rat (259). Pyridoxine probably affects fat synthesis through its effect upon the metabolism of the essential fatty acids, according to Witten & Holman (260). When pyridoxine was administered alone to rats deficient in both pyridoxine and essential fatty acids, the fat synthesis was much slower than when it was given with either linoleate or linolenate. Sinclair (261) states that a combined deficiency of both fat and pyridoxine produces more severe skin lesions and earlier death than does a deficiency of either, alone.

However, the two deficiency syndromes are distinct from one another. Sinclair (261) has never observed kidney lesions or hematuria in essential fatty acid deficiency, provided choline is supplied in the diet. Possibly, the kidney lesions observed by Panos & Finerty (251) may be related to lack of choline in the diet which they used.

Growth of certain *Neurospora* mutants requiring unsaturated fatty acids was studied by Lein, Puglisi & Lein (262). Their results indicate several points of difference between unsaturated fatty acid metabolism in *Neurospora* and the higher vertebrates.

VITAMIN D

New vitamins D.—Raoul and collaborators have recently described the isolation of antirachitic compounds which are distinct from vitamins D₂ or D₃ (263, 264). Two series of antirachitic compounds can be produced synthetically by appropriate treatment of cholesterol (265). Both series lead to substances (so-called "a" and "A"), which can fix calcium reversibly, which have antirachitic activity comparable to that of vitamin D in the rat, and which are closely related in structure to the classic D vitamins. Some of these antirachitic compounds have been shown to occur in the animal organism (264, 265). Further characterization of these compounds will be awaited with interest.

Assay methods.—Vitamins D₂ and D₃ can be separated from a mixture of cholesterol, 7-dehydrocholesterol, ergosterol, and sitosterol by chromatography on filter paper impregnated with stearato chromic chloride (266). Improvements in the chemical methods of determining vitamin D in foodstuffs and pharmaceutical preparations are discussed by Diemair & Mander-scheid-Schwindling (267) and by Wodsak (268). Shaw & Jeffries have described methods for the determination of ergosterol in yeast (269 to 272). The most accurate procedure involves saponification, extraction of unsaponifiable matter, digitonin precipitation, and subsequent spectrophotometric determination (270). Shorter methods of analysis (271, 272), as well as a method for preparing pure ergosterol (269), are also given.

Titus *et al.* (273) found that by pretesting parent stock they could breed chicks, which would have in response to a fixed quantity of vitamin D a tibia ash falling within a comparatively narrow range. By use of such chicks Kennedy *et al.* (274) could obtain significantly less error variance and greater precision of assay results in vitamin D bioassays, compared to results with unselected chicks.

Occurrence.—Rosenberg found no provitamin D in the preen glands of ducks, geese, or chickens but a relatively large amount in the sterols of chicken feet (275). He believes the natural provitamin D of birds is either 7-dehydrocholesterol or a sterol of very similar structure. Ward & Moore (276) detected, spectrophotometrically, a 7-dehydrosterol, presumably 7-dehydrocholesterol, in high concentration in certain sex organs of the male

and female rat. This sterol was not found in male sex organs of a number of other animals tested. Human sera were analyzed for content of 7-dehydrocholesterol by taking readings in the Liebermann reaction at 1 min. (277). Mean values for free and ester forms for 35 subjects were 3.4 and 16.5 mg. per 100 ml., respectively.

Balance studies.—An interesting balance study with rats fed a massive dose of vitamin D is reported by Cruickshank & Kodicek (278). Groups of weanling rachitic rats received an oral dose of 1 mg. of calciferol. On each of four succeeding days various tissues and excretory products were assayed biologically for vitamin D. About 23 per cent of the dose was accounted for, the fate of the remainder being unknown. On the first day the carcass, liver, intestines (minus contents), and feces accounted for, respectively, 1.7, 3.7, 0.6, and 14.3 per cent of the dose. By the second day all values except carcass vitamin D had decreased by at least one-half. No vitamin D was detected in the urine.

Hanahan & Wakil (279) showed that 2 to 5 per cent of orally administered ergosterol- C^{14} is absorbed by rats within 6 hr., mainly via the lymph system. Liver contains most of the absorbed ergosterol. Bile is a major excretory pathway for ergosterol metabolites.

Antivitamin D.—That extracts of green-feeds, when fed to rats on a rachitogenic ration, depress the bone ash response to a fixed dose of vitamin D was shown by Grant (280), who isolated and identified the factor as carotene.

Effect on calcium and phosphorus metabolism.—Carlsson *et al.* (281) carried out tracer experiments with Ca^{45} and P^{32} . The results suggest that vitamin D, in physiological amounts as well as in excess, promotes release of lime salts from the bones. Henry & Kon (282) found that vitamin D has no effect on calcium retention in the adult rat, whether the diet contains high (0.8 per cent) or relatively low (0.25 per cent) amounts of calcium.

Miscellaneous.—The coenzyme A content is a limiting factor in the capacity of living yeast to synthesize ergosterol, according to Klein & Lipmann (283).

The extent of blackening of feathers of buff varieties of chicks resulting from vitamin D deficiency was increased or decreased by selective breeding. However, both resistant and nonresistant lines were susceptible to rachitic effects of vitamin D deficiency [Lillie, Knox & Bird (284)].

Children with rickets excrete more of certain amino acids in the urine than do normal children (285, 286). Treatment with vitamin D reduces the excretion of these amino acids but not always to normal levels.

Warning against overdosage with vitamin D is still being given. The clinical picture caused by vitamin D intoxication is comprehensively described in two recent reports (287, 288). A state of hypervitaminosis D_2 was produced in mice by intraperitoneal injection of calciferol. Neither sulfhydryl compounds, rutin, or cortisone had any ameliorative effect on the hypervitaminosis (289).

VITAMIN K

Progress in identification of factors involved in blood clotting and papers concerned with clinical use of anticoagulant drugs are not covered in this review. Dam has reviewed recent studies on vitamin K (290).

Millar, Leddy & Fisher (291) followed the path of radioactivity after oral administration of radioactive menadione or vitamin K₁. Contrasting results showed about one-half of total administered radioactivity of menadione to be excreted in the urine with little or none passing through the intestinal lymph; while more radioactivity appeared in the lymph and less in the urine after dosage with C¹⁴-labeled vitamin K₁. Rats and dogs with intestinal lymphatic fistulas were used.

An adult dog requires 0.5 μ g. per day per kg. body weight of vitamin K₁; while a growing pup needs at least ten times as much. This suggests that the vitamin K requirement is markedly influenced by growth [Quick (292)].

At any value of prothrombin which is maintained by two different forms of vitamin K, the ratio of log dose of the two forms is a constant, according to Almquist (293). Interesting implications of this relationship and of data from chicks receiving bishydroxycoumarin are discussed by him. Dam & S ndergaard (294) compared the effects of intravenously injected vitamin K₁, menadione, and 2-methyl-1,4-naphthalenediol diphosphate, tetra-sodium salt, on the prothrombin time of chicks previously given bishydroxycoumarin. Vitamin K₁ caused the prothrombin time to decrease much earlier than did the other two compounds. After 22 hr., however, values in all groups had returned to normal. Prolongation of blood clotting time of chicks raised on practical rations can be caused by certain commonly used feed supplements; among these are terramycin and arsonic acid [Griminger *et al.* (295)]. The effect was reversible in each case, on adding menadione to the ration.

A new anticoagulant drug, 2-diphenylacetyl-1,3-indandione, appears to be 15 to 25 times as active on a weight basis as bishydroxycoumarin in man (296) and 200 times as potent in rabbits (297). Hamsters maintain normal prothrombin times even though they have blood plasma levels of bishydroxycoumarin well above the level (5 to 10 μ g./ml.) at which the prothrombin time in man is prolonged (298). Apparently, there are species differences in susceptibility to the action of anticoagulant drugs.

Addition of menadione to milk or to rations of lactating cows retarded the souring of milk; however, in long-term experiments the effect was variable [Kelley & Dittmer (299)].

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WATER-SOLUBLE VITAMINS, PART I¹

(FOLIC ACID, B₁₂, CF, CHOLINE, PAB, BIOTIN)

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In view of the continuing large output of research papers, the survey of the water-soluble vitamins is on this occasion divided into two parts. Part I will cover vitamin B₁₂ and intrinsic factor, folic acid, choline, *p*-aminobenzoic acid, and biotin. The entire field was reviewed to the end of 1952 in Volume 22 of the *Annual Review of Biochemistry*. To avoid delay in publication, it is only possible for the present review to include work published up to about mid-October 1953, i.e., less than a full year. Nevertheless, a considerable volume of new research has to be reported. Progress has been steady rather than spectacular and much remains to be discovered about the mode of action of these vitamins and even about the chemistry of the B₁₂ group.

VITAMIN B₁₂ OR CYANOCOBALAMIN

Allowing for the size of the molecule, progress with the chemistry of vitamin B₁₂ has been encouraging though tantalising. Controlled hydrolysis will remove the nucleotide, or a few moles of ammonia; the cobalt can be taken out by reagents so mild as ascorbic acid, provided the stabilising cyan group is first removed, but no recognisable product results. At the other extreme, vigorous oxidation yields products containing only a few carbon atoms. No known procedure yields fragments of intermediate size; for example, nothing approaching the elegance of the sulphite cleavage of thiamine has yet been reported. At the moment it almost looks as though the x-ray crystallographers might reach a solution before the chemists.

Schmid, Ebnöther & Karrer (1) oxidised a hydrolysis product of vitamin B₁₂ with alkaline permanganate and isolated eight crystalline acids from the mixture. They identified acetic, oxalic, succinic, methylsuccinic and dimethylmalonic acids. There were also four unidentified acids, namely: a monobasic of equivalent weight 221; a dibasic acid of equivalent weight 78 which readily formed an anhydride; another of equivalent weight 125 to 130; and another believed to be a substituted malonic acid. However, until some nitrogen-containing fragments are isolated one cannot get far with trying to piece the molecule together from these products. Catalytic hydrogenation of vitamin B₁₂ at 100°C. in *N* HCl liberated 5 moles of ammonia and a little methylamine arising from the CN group.

Work in the reviewer's laboratory and at the University of Cambridge [Armitage *et al.* (2)] has been concerned with the multitude of products arising

¹ The following abbreviations are used in this chapter: CF for *citrovorum* factor; CO for choline oxidase; FA for folic acid, IF for intrinsic factor; PGA for pteroylglutamic acid.

ing on controlled hydrolysis with acid or alkali. Electrophoresis on filter paper at pH 6.5 or 10 in the presence of cyanide affected separation into uniformly-spaced coloured zones attributable to classes of products containing one, two, three, etc. acidic groupings. One such series arising on prolonged standing with cold dilute mineral acids appeared to differ from vitamin B₁₂ only by the successive removal of ammonia from unsubstituted amide linkages. The most convincing evidence for this was the reconversion of the mono-, di-, and tri-basic acids in this series into vitamin B₁₂, identified by its microbiological activity, chromatographic and electrophoretic behaviour, and infra-red absorption spectrum. This was achieved by ammonolysis of the mixed anhydrides prepared by reacting the acids with chloroformic ester and triethylamine in anhydrous dimethylformamide. The mono- and di-carboxylic acids each separated on paper chromatography with *s*-butanol into unequal proportions of three isomeric acids; the tribasic acid could not be fractionated further. This is the behaviour to be expected from random hydrolysis of three amide groups at different rates. All these isomeric acids, and the tetrabasic acid that appeared in low yield on prolonged mild hydrolysis, were obtained crystalline. Repetition of the work of Schindler (3) indicated that the products he obtained by treatment of vitamin B₁₂ with alkali and hydrogen peroxide, were mixtures of these acids and unchanged vitamin B₁₂. The second series of acids, arising from somewhat more vigorous hydrolysis with hot mineral acids or alkali, had lost the nucleotide (dimethylbenzimidazole ribofuranoside phosphate) as well as successive molecules of ammonia. Still more vigorous hydrolysis removed aminopropanol and led to other changes resulting finally in products with 5, 6, and 7 acidic groupings. This series could be distinguished from the first by different behaviour on electrophoresis. The purple double cyanides of these compounds were stable not only in alkaline solution (as with vitamin B₁₂ and the corresponding acids) but up to about pH 4; however, the second CN group did not confer an extra negative charge as in the "B₁₂ series." In more acid solution, both CN groups were lost, and the compounds behaved as bases on electrophoresis in acetic acid, ionisation of carboxyl groups being suppressed. None of these nucleotide-free compounds was obtained crystalline. The neutral compound in this series, i.e., vitamin B₁₂ from which only the nucleotide had been removed, could be prepared in good yield by treating vitamin B₁₂ with concentrated hydrochloric acid, overnight in the cold or at 65°C. for 5 min. This compound still showed microbiological activity towards the B₁₂-requiring *Escherichia coli* mutant; indeed, by the agar plate assay technique its potency appeared to be about three times that of vitamin B₁₂, though it appeared lower by the turbidometric technique; the compound was inactive towards *Lactobacillus leichmannii*. Interest in this substance is enhanced by the observation that it displays the same microbiological, chromatographic and electrophoretic behaviour as "Factor B" extracted from calf manure by Ford & Porter (4). The same substance had also been extracted from a fermentation broth produced with a *Streptomyces griseus* strain. One could

speculate that the *E. coli* mutant was blocked at some step in the synthesis of this cobalt-containing part of the B₁₂ molecule but was able to synthesise the nucleotide and attach it to the Factor B to produce the required vitamin B₁₂, and indeed some evidence for this hypothesis has been obtained by Ford & Holdsworth (5).

The apparently enhanced activity of Factor B in the agar plate assay could be a result of the time required for this synthesis or to delayed uptake by the bacterial cells, permitting wider diffusion and larger growth zones of lower density than that produced by vitamin B₁₂ itself.

This paper (2) also confirms the findings of Cooley *et al.* (6), namely, that only one mole of isopropanolamine is present in the vitamin B₁₂ molecule, instead of two, as previously stated by Chargaff *et al.* (7). Cooley *et al.* used not only an improved version of the quantitative paper chromatography method of Chargaff *et al.*, but also estimated the aminopropanol, by the microdiffusion technique, from the ammonia liberated by periodic acid from an acid hydrolysate freed from other hydrolysis products. Armitage *et al.* (2) suggest that the aminopropanol is linked ester-wise to the phosphoric acid and amide-wise to a carboxyl group on the cobalt-containing part of the molecule. Hydrolysis of the latter linkage and of the unsubstituted amide groups (at least three) could lead to a red acid with at least four carboxyl groups. Along with ammonia arising from hydrolysis of the —CN group, at least 4 of the 5 or 6 moles of ammonia liberated could also be accounted for.

Controlled chlorination of vitamin B₁₂ with 3 moles of chlorine or with chloramine T has been described by Ellis *et al.* (8). The reaction mixtures can be fractionated into two or three purple compounds, respectively, by chromatography on paper. On repeated chromatography there is a tendency for "Compound CP-C" to pass into the second "Compound CP-B" and this again into the third "Compound CP-A." With excess cyanide all these compounds give deep blue solutions. Two atoms of chlorine were found per molecule; these compounds have neither microbiological activity nor antagonistic action towards vitamin B₁₂. On repeating this work in the reviewer's laboratory, it was found that some of these products could be fractionated further by electrophoresis. Schmid *et al.* (1) also obtained a violet microcrystalline product containing 3.8 per cent of chlorine by cautious treatment of vitamin B₁₂ in methanol solution with chlorine. This product also went blue with excess cyanide. Vigorous chlorination led to a product containing 30 per cent of chlorine.

Diehl *et al.* (9, 10) made the rather extraordinary suggestion that polarographic reduction of vitamin B₁₂ leads to a compound containing monovalent cobalt, and that of catalytically hydrogenated vitamin B₁₂ (vitamin B₁₂) to compounds containing cobalt of valencies 1 and 0. These claims have been refuted by Boos *et al.* (11), who criticise Diehl's interpretation of his polarographic data. They also point out that catalytic hydrogenation leads at best to a 70 per cent yield of product reconvertible to vitamin B₁₂, so

that in some of his experiments Diehl was using very crude products. They conclude from their own polarographic results that the brown compound arising on reduction of vitamin B₁₂ catalytically or with a chromous complex contains divalent cobalt, as might be expected; this compound is very readily reoxidised by atmospheric oxygen to a cobalamin containing trivalent cobalt. McConnel *et al.* (12) have reported work, mostly completed several years ago, on cobalt porphyrins and their absorption spectra in relation to that of vitamin B₁₂. They showed that several such compounds could coordinate with one or two moles of cyanide, or with one molecule of various benzimidazoles, or with both simultaneously. Meanwhile the x-ray crystallography group at Oxford make slow but steady progress with the interpretation of their results with cyano-, thiocyanato-, and selenocyanato-cobalamins. Three-dimensional models of much of the molecule have been constructed, and there is already clear evidence that, although a planar structure is present around the cobalt, it is certainly not a typical porphyrin structure [Hodgkin (13)]. This is in line with the failure of Armitage *et al.* (2) to obtain maleimides on controlled oxidation of the red acids arising on hydrolysis of vitamin B₁₂; it renders the more surprising, the claim by Bánhidi *et al.* (14) that certain cobalt porphyrins have B₁₂-like microbiological activity at concentrations around 100 µg. per ml., although cobalt mesoporphyrin is said to inhibit vitamin B₁₂.

Controversy continues as to whether any Co⁶⁰ or P³² radioactivity can be induced in vitamin B₁₂ by direct neutron bombardment, despite the ejection of most of the activated atoms through operation of the Szilard-Chalmers effect. Doubts are cast upon the claims of Anderson & Delabarre (15) and of Lester Smith (16), both by Numerof & Kowald (17) and by Woodbury & Rosenblum (18). In the later investigations nearly all the induced radioactivity was removed from the vitamin B₁₂. The reviewer contends that his own purification techniques were the most rigorous of those used in any of the investigations and is prepared to defend his claim. The matter is, however, of more interest to physicists than to biochemists, for the specific activity attainable is too low for biological investigations.

Dulaney & Williams (19) have attempted to throw some light on the biosynthesis of vitamin B₁₂. It is produced by *S. griseus* on a glucose and salts medium fortified with cobalt and phosphate. Tests with single amino acids as sole nitrogen sources, and with additions of complex natural products, carboxylic acids, purines, pyrimidines etc., failed to reveal any true precursor effect; that is to say, any increase in vitamin B₁₂ production ran parallel with increase in cell growth. Addition of the known portions of the molecule likewise failed to induce formation of extra vitamin B₁₂, but curiously, stimulation was caused by *o*-phenylene diamine, *o*-nitroaniline, *o*-xylydine, and 3,4-diaminotoluene. The use of deuterium-labelled *o*-phenylene diamine showed that it was not incorporated to any important extent into the B₁₂ molecule. Its effect in stimulating B₁₂ formation seems to be related to a slight inhibitory effect on the *S. griseus*.

Determination.—Little work has been published during the period under review. Fisher (20) has extended the applicability of the colorimetric method of Rudkin & Taylor (21) to dry microbial material by showing that vitamin B₁₂ can be extracted from it with hot benzyl or propyl alcohol in the presence of cyanide. Bardos & Gordon (22) have shown that hypertonic solutions of various salts inhibit growth of *L. leichmannii*, and that the effect can be reversed by vitamin B₁₂. Thus on raising the sodium chloride concentration from 1.1 per cent to 1.7 per cent the B₁₂ requirement for growth is increased 250-fold. The findings are consistent with the hypothesis that the vitamin combines with an apoenzyme and that the available concentration of the complex is controlled by the ionic concentration in accordance with Cohn's salting-out equation for proteins. The effect is not attributable to inhibition of cobalamin uptake by the cells (23). This work also shows that assays of samples rich in salts can be seriously in error. Campbell *et al.* (24) have made a thorough statistical analysis of results with a six-point assay design closely based on the U.S.P. 72-hr. titrimetric assay with *L. leichmannii*. They conclude that log dose versus log response gives a straight line plot and that limits of error of ± 5 per cent ($P=0.05$) are usually attained. Johansson (25) has made a similar thorough study of the turbidimetric assay with the *E. coli* mutant. The relative sensitivities of various lactobacilli used for the assay of vitamin B₁₂ have been investigated by Bánhidi *et al.* (26).

It is becoming clear from work on vitamin B₁₂-like factors (see later) that the *E. coli* mutant is the least specific of the available assay organisms. Ford (27) claims that *Ochromonas malhemensis* is the most specific for vitamin B₁₂. It does not respond to a protein-bound form of the vitamin occurring in sow's milk [Gregory *et al.* (28)], thus failing to realise the expectations of Hutner and colleagues (29), who found that certain chrysomonads require vitamin B₁₂ and who thought that these protozoa would probably resemble higher animals in being able to utilise bound forms.

Stability.—Rosenblum & Woodbury (30) describe a method using radioactive (Co⁶⁰) vitamin B₁₂ for assessing the stability of the vitamin in pharmaceutical preparations, such as multivitamin mixtures. The results obtained by this method, or indeed by any of the direct or indirect colorimetric methods, must, however, be accepted with caution for any deteriorated samples, in view of the evidence of Armitage *et al.* (2) that relatively mild conditions can produce degradation products almost indistinguishable from vitamin B₁₂ itself. Stapert *et al.* (31), using microbiological assay, find somewhat surprisingly that the cobalamin activity of liver extracts is stable on storage in full containers, but not when appreciable proportions of air are present. Loy *et al.* (32) on the other hand record losses of both vitamins B₁₂ and B_{12b} in the presence of some reducing agents, although both are stable (as is the cobalamin activity of milk and liver extract) in the presence of bisulphite, even on drastic heating at pH 3.5 to 9. Ostling & Nyberg (33, 34) find microbiological and clinical B₁₂ activity to remain in liver extract after heating at pH 10 for 1 hr. in a boiling water-bath. They interpret their findings in

terms of a new alkali-stable factor, but their data do not seem to rule out the persistence of part of the vitamin B_{12} , especially since it is known to be protected from such relatively mild alkali treatment by unknown components in some crude sources such as liver extract [see for example Guttman & Vandenheuvel (35)]. Campbell *et al.* (36) have independently confirmed the work of Frost *et al.* (37) by showing that vitamin B_{12b} , but not B_{12} itself, is destroyed by heating with ascorbic acid at pH 5; this method cannot be used to differentiate between the two cobalamins in liver extracts, because the iron which is usually present exerts a protective action.

Intrinsic factor.—Castle's intrinsic factor continues to occupy the attention of several research groups, but the knotty problems of its nature and mode of action remain unsolved. Indeed evidence accumulates in favour of both of two incompatible hypotheses! It is generally agreed that IF¹ combines with vitamin B_{12} to form a heat-labile complex that is nondialysable, inactive for B_{12} -requiring microorganisms, incapable of being taken up by B_{12} -absorbing microorganisms, and orally effective in pernicious anaemia by promoting absorption from the gut. Unfortunately other substances share all but the last of these properties with the intrinsic factor, so that assays based thereon, though they may be precise, are of uncertain significance, while clinical assays, though specific, are hopelessly crude. The assay method of choice is probably to measure radiometrically the absorption by the gut of a pernicious anaemia case (in remission) of a minute dose of Co^{60} - B_{12} , taken with the test preparation (38). This method is giving promising results in several laboratories in England as well as in the United States. However, Meyer (39) reports a bigger range than others have found among normal subjects. Moreover although pernicious anaemia cases in remission gave the usual high faecal elimination values, these were not much reduced on giving extracts of hog gastric mucosa (Ventriculin) with a second test dose of Co^{60} - B_{12} . It is open to question whether this test can be valid for a crude IF preparation that probably already contains bound B_{12} . Hoff-Jørgensen and co-workers (40, 41, 42) and Burkholder (43) regard the principal function of intrinsic factor as prevention of B_{12} uptake by the coliform bacteria invading the upper intestine and even the stomach of achlorhydric pernicious anaemia cases. Others consider that the complex is for some unspecified reason more easily absorbed than in the free vitamin; alternatively it has been suggested that combination is more or less irrelevant and that the IF somehow conditions the gut wall, rendering it more "permeable" to the vitamin. In favour of this view may be cited the fact that serum B_{12} is in a bound form and that blood will bind added B_{12} : however, the binding factor in blood is not necessarily the same as that in gastric juice and mucosa. Horrigan & Heinle (44) believe they can explain an odd case of refractory macrocytic anaemia by a deficiency of the plasma binding factor. Wallerstein *et al.* (45) find that gastric juice does not potentiate vitamin B_{12} when the mixture is given by injection, i.e., the complex does not function as an "activated vitamin B_{12} ." They also claim in confirmation of Glass *et al.* (46) an inverse relationship be-

tween clinically effective doses of IF and vitamin B₁₂; whereas 1 µg. vitamin B₁₂ plus 10 ml. gastric juice daily was almost ineffective, increasing the dose of either component five-fold caused a response. Moreover, the IF dose was effective if given a few hours before the vitamin B₁₂ but not if given at the same time interval after the vitamin dose. For the rival hypothesis is cited the facts that in achlorhydric conditions bacteria capable of absorbing relatively large amount of vitamin B₁₂ certainly do invade the upper intestine. This uptake by bacteria is thought to prevent subsequent utilisation by the host; certainly pernicious anaemia cases eliminate in the faeces large amounts of vitamin B₁₂ that they cannot utilise, but it is synthesised mainly in the colon, from which absorption would not be expected to occur. One can bring the argument around full circle by pointing out that in any event the pernicious anaemia subjects lack intrinsic factor to promote any such uptake. In other words, all the many investigations in this field have still not brought us far beyond the guessing stage, and the really critical experiment remains to be devised. It may be noted that vitamin B₁₂ synthesised by gut organisms is the only evident source available to complete vegetarians, and omnivorous feeders may possibly supplement from this source the rather meagre intake provided by a normal diet. Broberg *et al.* (47) estimate the average intake of a small group of normal women as only 1.1 µg. per day, compared with a faecal elimination of 10 to 60 µg. per day. From the values quoted later for the vitamin B₁₂ content of milk (84, 85, 86), one would anticipate a higher daily intake unless the diet contained unusually little milk and milk products.

Additional evidence for the bacterial uptake idea also comes from the favourable effect that the antibiotics have sometimes had on macrocytic anaemias (48 to 51). Morgensen's report (51) of sudden increases in new cases of pernicious anaemia in two districts in Denmark also suggests infection as a contributing factor. If the hypothesis is right, then the bacterial uptake method is a valid assay technique for IF. In support of this, Hoff-Jørgensen claims that it gives results in agreement with clinical tests, in indicating the location of IF in various parts of the hog stomach and intestine (41) and in guiding fractionation procedures (42). On the other hand Chow & Davis (52) have shown that nucleic acid and heparin preparations also prevent uptake of vitamin B₁₂ by bacteria, yet these substances have not been shown to have clinical efficacy. Swendseid *et al.* (53) have found by the tracer technique that absorption of oral vitamin B₁₂ is seriously impaired after gastrectomy unless gastric juice is also given. Yet the same workers (54) find that the duodenal secretion of normal subjects, though not of pernicious anaemia cases, contains about the same amount of binding factor as does gastric juice. If this duodenal secretion had true IF activity, however, one would have expected it to promote B₁₂ absorption by the gastrectomy cases. One must continue to accept with caution results based on assessment of binding power, by whatever technique. Hoff-Jørgensen (41) finds the highest concentrations of binding factor in the pyloric mucosa and duodenum prox. in the hog.

Meulengracht (55) finds some IF activity in pyloric musculature as well as in the mucosa.

Prusoff and co-workers (56) have published in more detail their fractionation procedure comprising fractional precipitation by ammonium sulphate of a saline extract of dried hog stomach. After removing an inactive precipitate at pH 1.5, the extract was brought to pH 4.5 and cooled to 5°C., and ammonium sulphate was added. Fraction A precipitated at 35 per cent saturation, contained 28 per cent of the protein in the solution, and had little IF activity. Fraction B which came down between 35 and 55 per cent saturation, contained less than 2 per cent of the protein but a considerable proportion of the IF activity. Fraction C precipitated by full saturation with ammonium sulphate had 70 per cent of the protein but little activity. Fraction B showed clinical activity in pernicious anaemia at daily doses of 13 mg. (derived from 50 gm. Ventriculin) along with 5 μ g. of vitamin B₁₂. This quantity of Fraction B could bind only 0.2 μ g. of vitamin B₁₂ measured by the microbial inhibition method, or 2.2 μ g. measured by the dialysis method of Bird & Hoevet (57). However, during dialysis of this fraction, separation occurred into a soluble and an insoluble portion; both were active in pernicious anaemia, but the insoluble part had negligible binding power. Doubt is expressed whether pure IF will show any B₁₂-binding power at all. Fraction B was known to be far from pure, since electrophoresis revealed seven components.

Other significant advances in the fractionation of intrinsic factor have resulted from the use of electrophoretic techniques. Roland *et al.* (58) have used the old multicell apparatus in which a high potential applied to a relatively unbuffered system creates a pH gradient causing isoelectric fractionation of protein components. The B₁₂-binding protein of hog duodenum extract appeared to be isoelectric at pH 4.6. Latner *et al.* (59, 60) have used ionophoresis on buffered filter-paper to fractionate vitamin B₁₂ bound *in vivo* or *in vitro* to serum proteins, and also to fractionate gastric juice on a preparative scale. Considerable concentration was effected by ultrafiltration, and the material was then applied to thick filter-paper: after the ionophoresis at pH 6.4, sections were cut out while still wet and extracted by centrifuging. The fractions were then tested individually for protein and for binding factors and were combined appropriately for clinical tests. Most of the protein moved towards the cathode, and the results showed one or more peaks of binding activity not associated with IF activity. Material in the "anode peak" was active by both tests. A later paper (61) reports the preparation in this way of IF showing activity by the tracer technique in a dose of only 2 mg. The product appeared to be electrophoretically homogeneous and to have the properties of a mucoprotein. Marmion *et al.* (62) point out that reception-destroying enzyme, which is a mucinase, does not inactivate IF; however, this is not positive proof that IF is not a mucoprotein. Latner & Ungley (61) are sufficiently confident of the purity of their product to mention that analyses for component sugars and aminoacids are in progress.

Assuming, however, that it combines mole for mole with vitamin B₁₂, then the molecular weight of this protein would appear somewhat large, suggesting that complete purification may not yet have been achieved. On the other hand, Chow & Yamamoto (63) suggest that the binding factor of gastric juice may have a molecular weight as high as 5×10^6 . They also suggest that it contains a strongly acidic group that may be concerned in the linkage with vitamin B₁₂ [cf. (52)]. Schilling & Deiss (64) have studied a mixture of dialysed gastric juice and Co⁶⁰-B₁₂ by paper electrophoresis at pH 8.6. Radioactivity was concentrated into an anode region apart from the main protein peaks. Gregory *et al.* have also applied ionophoresis on paper for the final purification of a binding factor present in sow's whey (28). Holdsworth (65) has adapted Durrum's technique (66) of continuous electrophoresis on a sheet of filter paper to the preparations of this factor in a state approaching purity. Its binding power for several factors related to vitamin B₁₂ is the same, on a molar basis, as that for cyanocobalamin itself (67). Unfortunately this factor has not yet been assessed clinically: it has properties in common with those of IF but appears to be more heat-stable. There has been little speculation on the chemical bonds involved in B₁₂ binding. Cooley *et al.* (68) have suggested direct linkage between histidine and cobalt to form a "cobalichrome." This is conceivable with the naturally occurring bound forms, but seems unlikely as an explanation of spontaneous binding on mixing in dilute solution, because displacement of the firmly-linked —CN group is involved. Probably the only group that could co-ordinate with the cobalt without displacing—CN (i.e., by displacing the benzimidazole instead) is the—SH group. The strongly acidic group of heparin, and according to Chow (63) of the IF also, might form salt links with the feebly basic groups present in vitamin B₁₂, but one would expect alkali to dissociate such a complex. Hydrogen bonding is another possibility, but whatever the linkage is, it is firm enough to resist prolonged dialysis, which would tend to remove continuously any free vitamin in equilibrium with the bond form. Finally, since the heat-labile IF may well be an enzyme, the formation of a peptide bond is conceivable, arising perhaps by exchange of ammonia for the protein, on one of the —CONH₂ groups of vitamin B₁₂ (2). Spies *et al.* (69) have reported on the clinical value of a commercial preparation of vitamin B₁₂ with IF. As might be expected it was less effective in nutritional anaemia and sprue than in true pernicious anaemia. They claim, surprisingly in the light of much previous work, that the oral activity of the preparation is not destroyed by boiling for 30 min. The oral efficacy of very large dose of vitamin B₁₂ given without IF has received further confirmation in a recent report (70), which states that 20 pernicious anaemia cases have been satisfactorily maintained in this way for 3½ years or longer.

Therapeutic uses.—The antianaemia Advisory Board of the U.S.P. (71) has now recommended the assessment of injectable liver extracts by microbiological assay, equating 20 µg. of vitamin B₁₂ per ml. with 15 U.S.P. units. Retention of the clinical test is recommended for oral preparations. It sug-

gests that there is no advantage in using injectable solutions stronger than 20 $\mu\text{g.}$ per ml. whether of vitamin B_{12} or liver extracts, because urinary excretion causes wastage of higher doses. This very conservative recommendation does not tally with the experience of many haematologists who use relatively high doses of vitamin B_{12} for initial treatment of relapsed cases, especially when subacute combined degeneration of the spinal cord has set in; moreover, over half the dose is usually retained when amounts up to 100 or even 200 $\mu\text{g.}$ are given by intramuscular injection. Mollin & Ross (72) have continued their exhaustive studies of the state of the blood and bone marrow during treatment of pernicious anaemia cases. Serum B_{12} assays by the sensitive Euglena method have been supplemented by repeated bone-marrow examinations: reversion of the marrow towards a megaloblastic condition is the first sign of inadequate dosage with vitamin B_{12} . They point out that a normal individual stores some 1000 to 2000 $\mu\text{g.}$ of vitamin B_{12} in the liver and in other tissues, but that these reserves are thoroughly depleted in pernicious anaemia. They recommend, not unreasonably, that these reserves ought to be built up again by giving say five injections of 1000 $\mu\text{g.}$ during one or two weeks, from each of which 200 to 300 $\mu\text{g.}$ would be retained. After the more usual modest dose, the serum level of vitamin B_{12} soon falls below the lower limit for normal subjects (100 $\mu\text{g.}$ per ml.), at about which point the marrow becomes megaloblastic again. As the authors imply, we should not be surprised at this, but rather at the fact that small doses are so well mobilized for hemopoiesis, despite the state of depletion in the tissues. Reports continue to appear on long-term maintenance of pernicious anaemia cases: usually vitamin B_{12} or a mixed cobalamin concentrate is found just as satisfactory as liver extract, though occasional "refractory" cases turn up. Meacham & Heinle (73) found an average dose of 1 $\mu\text{g.}$ per day, given at intervals of three to four weeks, satisfactory for most of their 43 cases, though in some a mild macrocytosis persisted not affected by liver extract or folic acid; the possibility that the B_{12} requirements of these patients was abnormally high seems to call for exploration before a new missing factor is postulated.

Reisner & Weiner (74) have made a further study of the effects of massive intramuscular injections of vitamin B_{12} . A single 1 mg. dose effected, in all but 1 of 14 cases, remissions, lasting three to nine months. Several patients with subacute combined degeneration, stabilised by prolonged conventional treatment, showed little or no further improvement on 1 mg. of vitamin B_{12} weekly; 51 to 98 per cent of the 1 mg. dose appeared in the urine within 48 hr. It is concluded that massive doses cannot usefully be substituted for more frequent smaller doses; one cannot help thinking that some patients might welcome the opportunity of reporting to their doctors only three or four times a year, but the intensive therapy may not be entirely without risk, because one interesting case was thought to have developed a true polycythemia after a 1 mg. dose. Another case of temporary polycythemia, and also splenomegaly, presumably attributable to vitamin B_{12} , resulted from giving a gouty patient only 80 $\mu\text{g.}$ daily by mouth with hog stomach ex-

tract [Kjerulf-Jensen & Schwartz (75)]. Monto *et al.* (76) report the successful treatment of six pernicious anaemia cases by inhalation of pure vitamin B₁₂ in saline or in lactose dust, but it is not clear what advantage is claimed for this route. In subacute combined degeneration of the spinal cord, accompanying severe pernicious anaemia, Earl *et al.* (77) found abnormally high levels of blood pyruvate; these returned to normal on administration of vitamin B₁₂. The conflicting claims for the value of oral vitamin B₁₂ for under-developed children, considered in previous reviews, have recently been critically and somewhat unfavourably re-evaluated (78). Similarly, early hopes that vitamin B₁₂ would prove to be of great value in other conditions seem to be fading: it proved useless, for example, in 25 cases of acute leukemia [Welsh (79)]. However, favourable reports on the use of massive doses in various neurological conditions continue to appear, e.g., that of Lereboullet & Pluvineau (79a). Findlay (80) suggests from the results of experiments on rats that vitamin B₁₂ may help in the early phases of wound healing by increasing the tensile strength. However, one proven use has been found for vitamin B₁₂; it can serve in massive dosage as an antidote to cyanide poisoning, avidly taking up cyanide to form the nontoxic cyanocobalamin [Mushett *et al.* (81)].

Animal nutrition.—The place of vitamin B₁₂ in animal nutrition being well established, interest has turned to finer details, such as its effect on reproduction and lactation. Daniel *et al.* (82) have studied reproduction in rats on a vegetable diet with sulfasuxidine. They find that added vitamin B₁₂ is transferred to the young prenatally, and is present in the milk, especially during the first two days after parturition. Litters are heavier with B₁₂ supplementation but not up to the weights of those on the stock diet, while the second and third pregnancies on this diet give poor litters, suggesting deficiency of some unknown factor. Dryden *et al.* (83) find that lactating rats produce less milk when in a state of B₁₂ deficiency, but this may be an indirect effect of lower food consumption. According to van Koetsveld (84) the vitamin B₁₂ content of milk rises from an average of 6.3 µg. per l. during the last week the cows are indoors, to an average of 10.1 µg. per l. during their first week on pasture. These values are much higher than those quoted by Collins *et al.* (85), namely 2.6 to 4.4 µg. per l. for market or pasteurised milk, with no significant seasonal variation. Collins *et al.* (85, 86) also find, for both cows and goats, a higher vitamin B₁₂ content in the colostrum than in the regular milk; mineralised goats milk was too low in vitamin B₁₂ to induce optimal growth of rats (87). Woolley (88) has claimed that spontaneous mammary tumors in three strains of mice are capable of synthesising vitamin B₁₂. The alleged phenomenon was detected through better growth of the suckling young of these tumorous animals when all the does were kept on a B₁₂-deficient diet; transplanted tumors were ineffective. Jaffé (89) finds that not only increased dietary protein, but also addition of 5 per cent of urea to the diet of B₁₂-deficient rats impairs reproduction compared with that of animals on a normal nitrogen intake. Frost *et al.* (90) describe an improved

rat growth assay for vitamin B₁₂; results on liver extracts parallel microbiological assays. Transfer of vitamin B₁₂ from the food of the hen to the egg and the chick has received some attention. Halick *et al.* (91) find that much higher B₁₂ levels in egg yolk result from injection of the vitamin than from feeding it. Thus with doses of 200 µg. per day, injection gave a 100-fold increase if B₁₂ in the yolk, but feeding only a 6-fold increase. This is reasonably interpreted as indicating limited absorption of oral B₁₂ or possibly destruction. Absorption of high levels (1750 µg. B₁₂ per kg. diet) but not of low levels (30 µg. per kg. diet) was raised about 30 per cent by adding a surfactant to the diet. Hatchability was not adversely affected by the abnormally high vitamin contents (up to 1 µg. B₁₂ per gm. of yolk) reached in some eggs. Jackson *et al.* (92, 93) find that radioactive vitamin B₁₂ injected into eggs is largely retained by the chicks, 30 per cent being still present after 12 weeks; the growth response also persists for at least this period.

Kawazu disease is a condition occurring in cattle in Japan, characterised by a macrocytic anaemia cured by injecting vitamin B₁₂. It is believed to be caused by SiF₄ ions in the water inhibiting the multiplication of B₁₂-producing actinomycetes, which were absent from the faeces of afflicted animals. Administration of silicofluoride to calves brought on the disease within one month, and in ten months the red cell count was halved [Sahashi *et al.* (94, 95)]. Cartwright *et al.* (96) induced a macrocytic anaemia in swine by withdrawing vitamin B₁₂ and folic acid and adding succinylsulfathiazole and a folic acid antagonist to the diet. No neurological disturbances could be observed. They found that, whereas either vitamin B₁₂ or folic acid stimulated growth, both were needed to cure the anaemia.

Ritter *et al.* (97) find that coprogen, an iron-containing pigment active as a growth factor for *Pilobolus*, has erythropoietic activity. In rats and chicks in which bone marrow degradation has been induced by folic acid antagonists or sulfaguanidine, coprogen in doses around 1 mg. per day stimulates erythrocyte formation. McGinnis *et al.* (98) present preliminary evidence for two new unidentified chick growth factors, present respectively in fish solubles and fermentation products. Tria & Barnabei (99) make a somewhat unconvincing claim to have demonstrated in an acetone extract of liver a new factor that enhances the growth of yeast cells and rats. One "new factor" has, however, been laid to rest: the methanol-soluble mink factor turns out to be vitamin B₁₂ [Leoschke *et al.* (100)].

The addition of antibiotics to feeding-stuffs is increasingly practiced, not only in the U.S. but now also in the United Kingdom. They have definite value for young ruminants, as well as for pigs and poultry [Knodt (101); Kon *et al.* (102)]. A recent review shows, however, that the precise modes of action are no better understood than they were a year ago [Stokstad (103)]. Attempts to find if they affect intestinal synthesis of vitamins have given conflicting results. Chow *et al.* (104) found that aureomycin led to increased faecal excretion of vitamin B₁₂ by rats, though other antibiotics had little or no effect, whereas Johansson *et al.* (105)

found no effect with aureomycin. Johnson *et al.* (106), however, found by a tracer technique definite evidence for increased intestinal production of thiamine on feeding penicillin. Catron *et al.* (107) claim that for swine aureomycin has a sparing effect on both pantothenic acid and vitamin B₁₂, and that these show mutually sparing actions in the absence of aureomycin.

Metabolism and Excretion.—The pattern of excretion of vitamin B₁₂ by man and various animals is now well established, and there is little new work to report. The findings do not, however, seem to have been co-ordinated into a logical hypothesis. It is clear from use of the tracer technique that absorption of small but nutritionally adequate amounts from the gut is quite efficient, except in the absence of IF as in pernicious anaemia: the picture has become confused through emphasis on the poor absorption of relatively large doses. Moreover, there has been a tendency to suggest that absorption may be negligible after large oral doses because little or no increase in urinary excretion can be detected. This seems to be a false argument: it is known that the vitamin occurs in a bound nondialysable form in liver and in blood, and that binding must probably precede oral absorption. In any event, absorption from the gut will be slow enough to permit binding before the material reaches the kidney, and urinary excretion of a protein-bound substance is not to be expected. After intramuscular injection of a sufficiently small dose, of the order of a day's requirements of the vitamin, the pattern is similar to that after oral administration, that is to say, only a very small proportion appears in the urine, presumably because there is time for binding to occur. Again, attention has been focussed on the "abnormal" pattern after relatively large doses, when increasing proportions of the dose appear in the urine as the size of the dose is increased. This is surely a result of a kind of spill-over of the free vitamin in excess of the capacity of the blood either to bind it or to transport it to storage depots [see Lester Smith *et al.* (108); Girdwood (109)]. It seems significant that excretion of even a massive dose virtually ceases after 8 hr.; what is then still retained is firmly held for a long time. Thus any idea of a saturation test for vitamin B₁₂ becomes meaningless; a suitably devised dosage schedule could presumably build up enormous and entirely abnormal reserves. Certainly a modest dose of radioactive vitamin B₁₂ given to a pernicious anaemia case in relapse was tenaciously retained, and deposits in the liver and spleen could still be detected months later, despite repeated injections of the unlabelled vitamin [Lester Smith *et al.* (108)]. This study also demonstrated rapid concentration of activity into the bone marrow, to a level several times that in simultaneously taken blood samples. Prolonged retention of labelled vitamin B₁₂, by rats, has been noted by Harte *et al.* (110); as in human subjects flushing doses induced only slow elimination. Chow and his colleagues have shown that in man, though not in rats, there is a gradual change with age in the proportions of injected doses that are eliminated in the urine; retention increases with age (111, 112). Radiometric measurements may exaggerate the small urinary excretion after oral administration. Studies with vitamin B₁₂ labelled with both P³² and Co⁶⁰

showed that in rats, part, and in a human subject, most, of the radioactivity in the urine was not attributable to microbiologically active cobalamin; the rest was presumably attributable to degraded vitamin B₁₂ though surprisingly the P*/Co* ratio in the urine was virtually the same as in the dose [Lester Smith (113)].

Biochemistry.—Interest continues unabated in the metabolic functions of vitamin B₁₂. If it participates in a single enzyme system then it is hard indeed to see how all the other functions attributed to the vitamin could follow on as secondary effects. However, any such speculations must start from one fact that now seems thoroughly established, namely that vitamin B₁₂, as well as folic acid, is concerned in the metabolism of one-carbon fragments; this might indeed provide a clue to the partial interchangeability of the two substances in correcting impaired haemopoiesis. It now seems likely that vitamin B₁₂ does not control transmethylation, i.e., the movement of preformed labile methyl groups from one acceptor to another, but rather the actual *de novo* synthesis of labile methyl groups. This is strongly suggested by the work of Kratzer (114) and also of Stekol *et al.* (115). They find, in poult and chicks respectively, that methionine synthesis from homocysteine and betaine can proceed in the absence of vitamin B₁₂. The high mortality of poult in B₁₂ deficiency suggests, however, that it has other functions besides bringing about synthesis of labile methyls. The carbon of glucose is available for synthesis of these methyl groups, appearing in methionine, choline, and creatine (115). This may link up with the evidence of Ling & Chow (116) that vitamin B₁₂ is concerned with the metabolism of carbohydrates and of lipids but not of protein. In *E. coli* mutants also, vitamin B₁₂ appears to mediate methyl synthesis [Kalan & Ceithaml (117); Dubnoff (118)]. A very thorough study of the precursors of the labile methyl group has been made by Arnstein & Neuberger (119). The hydroxymethyl group of serine appears to be the most important precursor, and formate next, the α -carbon of glycine being relatively inefficient. Vitamin B₁₂ stimulated methyl-group synthesis from all three precursors. Incorporation into methionine appeared to be the initial step followed by transmethylation of choline. These conclusions are confirmed in part by Stekol *et al.* (120, 121). The body contains a small pool of cyanide, in equilibrium with the cyan group of vitamin B₁₂. Boxer (122) has shown that the carbon of cyanide and thiocyanate contributes to a limited extent to synthesised methyl groups, probably via formate as intermediate. On the other hand Williams *et al.* (123, 124), from their study of liver enzymes in B₁₂ deficiency states, still regard the vitamin as being concerned in transmethylation. Moreover, fresh evidence is forthcoming to show that under rigidly controlled experimental conditions it is after all possible to demonstrate an effect on protein utilisation and nitrogen retention [Cheng & Thomas (125); Rupp & Paschkis (126)]. Also Hsu *et al.* (127) find the plasma proteins higher in chicks given vitamin B₁₂ than in deficient controls. They suggest that the vitamin may function in protein metabolism by stimulating the formation of ribonucleoproteins and plasma proteins.

Dubnoff (118 and earlier papers) has claimed a role for vitamin B₁₂ in the reduction of —S—S— compounds to the —SH state, and this idea has lately gained support. Thus Ling & Chow (128) find that the SH content (mainly as glutathione) of rat red blood cells falls during a deficiency of vitamin B₁₂ and rises on its administration. This is paralleled by a fall in blood SH in pernicious anaemia which on treatment rises, overshoots the normal level, and then returns to normal. They point out that glutathione may well reactivate the SH enzymes concerned with carbohydrate and lipid metabolism, and with general cellular proliferation and growth. The idea that vitamin B₁₂ is concerned in the reduction of —S—S— compounds is taken a step further by the recent announcement (70) that in absence of the vitamin coenzyme A may fail to be reduced to the —SH form, thereby inhibiting the normal pathway of fat metabolism. It is found, in support of this hypothesis, that in chronic deprivation of vitamin B₁₂, fat accumulates in the tissue of both chicks and rats. The work of Stekol *et al.* (120) also suggests a connection between coenzyme A and vitamin B₁₂; deficient rats have a reduced capacity to utilise C¹⁴-acetate for the acetylation of α -amino- γ -phenylbutyric acid, indicating impairment of the function governing the use of acetate for acetylation. Anderson & Stekol (129) have studied in rats the biosynthesis of glutathione from labelled precursors. They concluded that neither B₁₂ nor folic acid were concerned in the synthesis of glutathione from its amino acid components, but that both were involved in the incorporation of carbon-2 of glycine into both the cysteine and glutamic acid moieties. By way of adding to the multitudinous roles of vitamin B₁₂, High & Wilson (130) record its effect on increasing tissue deposition of vitamin A after carotene feeding. There is no effect on utilisation of preformed vitamin A, so the phenomenon involves improvement either in the absorption of carotene or more likely in its conversion to vitamin A. They suggest that this may be an indirect result of the involvement of vitamin B₁₂ in choline synthesis and of choline in the conversion of carotene.

Antagonists.—Interest in the idea of preparing compounds that might inhibit the biosynthesis or utilisation of vitamin B₁₂, has been heightened by Woolley's claim that mammary tumours can synthesise the vitamin (88, 131). However, there is negligible progress to report. Beiler's alleged antagonist made by treating vitamin B₁₂ with hydrogen peroxide and hydrochloric acid (132) seems to have been accepted at face value, though it was without effect in other systems (123, 133). It is in fact a chlorination product, and the reviewer's colleagues have found it to have no inhibitory effect in microbiological tests either, when the residual hydrogen peroxide was destroyed.

Controlled chlorination was also examined by Ellis *et al.* (8) in the vain hope that it might lead to an antagonist. 5,6-Dimethylbenziminazole, which acts as a feeble inhibitor, is found to interfere with the uptake of Co⁶⁰-B₁₂ by cells of the *E. coli* mutant grown with the vitamin, but not by cells grown with methionine [Oginsky & Smith (134)].

Tamm *et al.* (135, 136, 137) have found a range of alkyl benziminazoles

that inhibit the multiplication of the influenza virus, but this effect was not reversed by vitamin B₁₂, so that the structural similarity to part of the molecule is probably fortuitous.

Cobalt deficiency.—It now seems fully accepted that ruminants need cobalt primarily, if not exclusively, for rumen synthesis of vitamin B₁₂, and that when their reserves are depleted they cannot utilise small doses so efficiently as can the deficient human. There is little to add to a recent review of this subject (138). Rothery *et al.* (139) have studied the distribution of radiocobalt in the organs of the sheep after prolonged feeding. Davis *et al.* (140) found that, in the absence of B₁₂, cobalt slightly improves chick growth, presumably by promoting intestinal synthesis of the vitamin.

FACTORS RELATED TO VITAMIN B₁₂

A number of vitamin B₁₂ analogues are now known; they arise from rumen or intestinal synthesis or from other fermentations. They have been here relegated to a separate section, because it now seems that they are of use neither to man nor beast, and to a few species of bacteria only, so that it is doubtful if they merit the designation "vitamin." Their nomenclature is indeed in a chaotic state; one substance has received at least three names and most of the specimens available have proved to be mixtures. The different factors appear to be minor variants of a large molecule, so that to isolate and purify them the utmost refinements of preparative technique are called for. Already we have come across a pair of factors so difficult to separate that the possibility of others proving altogether inseparable by presently known methods must be envisaged. Our knowledge of these factors has been brought to a reasonably ordered state by workers at the National Institute for Research in Dairying (Shinfield, England). The most powerful technique applicable is electrophoresis on filter paper, especially in acetic acid, when separation presumably depends on differences in basic strength. It can be used analytically in conjunction with bio-autographic delineation on a submicro scale. The zones of growth can be rendered more plainly visible by including triphenyl tetrazolium chloride in the agar medium; this causes them to show up as cloudy deep red areas [Ford & Holdsworth (141)]. Alternatively, electrophoresis can be used to give directly visible zones, with quantities above 20 μ g. or so, and also for the final stages of purification on a small preparative scale. Repeated electrophoresis is necessary to obtain pure specimens. This method can usefully be supplemented by chromatography on paper. Holdsworth (142) and Ford *et al.* (143, 144) have shown by these methods that the major component in their Factor A and in vitamin B_{12m} [Wijmenga (145)], is the same substance, which is different from pseudo-vitamin B₁₂ [Piffner *et al.* (146)], though samples were mutually contaminated and usually contained some vitamin B₁₂ also. Vitamin B_{12t} [Lewis *et al.* (147)] is probably also a mixture of these two substances. Factor B is distinct: as mentioned earlier, it is in fact vitamin B₁₂ lacking the nucleotide, and its basicity in acetic acid solution is akin to that of vitamin B_{12b}, i.e., it arises from loss

of the cyan group in acid solution. Factor C, which has acidic properties, can also be clearly differentiated from the others. It will be recalled that pseudo-vitamin B₁₂ contains adenine in place of the 5,6 dimethylbenziminazole of vitamin B₁₂ [Dion *et al.* (148)], and it presumably owes its weak basicity to the purine 6-amino group. The nucleotide part of Factor A also contains an amino group. The reviewer has found (unpublished work) that both factors can be deaminated with nitrous acid to yield new crystalline neutral factors separable chromatographically, but not electrophoretically, from vitamin B₁₂ and still microbiologically active. He has also demonstrated that on treatment with warm concentrated hydrochloric acid (2) they both yield a substance indistinguishable from Factor B. Thus vitamin B₁₂, pseudo-vitamin B₁₂ and Factor A differ solely in the nucleotide parts of their molecules. These observations afford some clue to the biogenesis of this large family of factors. It would seem that many microorganisms can incorporate cobalt into the "red part" of the molecule to yield Factor B. Some can take the synthesis no further; others can complete it to yield vitamin B₁₂ itself, whereas again others add on instead adenylic acid or some different nucleotide. It appears indeed that *E. coli* can direct the synthesis along alternative routes, probably depending on the abundance of the various nucleotides present, for Ford *et al.* (5, 149) have shown that *E. coli* can convert the starting material, Factor B, into several of the other factors. The situation may be likened to that in which *Penicillium chrysogenum* will incorporate some aliphatic acids and many substituted phenylacetic acids into the side chain of penicillin, to give a family of penicillins. Recently Smith & Brown (149a) have found that Factor A contains 2-methyl adenine in the nucleotide part. Holdsworth (5) has confirmed this characterisation by showing that *E. coli* will produce mainly Factor A if incubated with Factor B and 2-methyl adenine. Deaminated Factor A was shown to contain the anticipated 2-methyl hypoxanthine. This deaminated Factor A and also deaminated pseudo-vitamin B₁₂ (containing hypoxanthine) were both extracted in trace amounts from pig faeces. There seems no reason why still more B₁₂ factors on this general pattern should not turn up, and indeed unidentified minor spots are often to be seen on bio-autographs of crude preparations. Moreover there is no *a priori* reason why all these factors should have a part to play in the metabolism of higher animals. Indeed it now seems probable that any activity they appeared to have for rats, chicks, or man [Hawk & Elvehjem (150); Chalmers (151)], was a result of small amounts of contaminating vitamin B₁₂. What is more, they are probably not even absorbed from the alimentary tract or at any rate they are not stored in the liver except in traces [Ford *et al.* (152)].

FOLIC ACID, FOLINIC ACID OR CITROVORUM FACTOR, AND RELATED COMPOUNDS

After the interest created last year by the announcement of the synthesis and resolution of folinic acid, research activity in this field seems to

have abated somewhat. This review is only intended to supplement those published in the preceding numbers of the *Annual Review of Biochemistry* by covering new work published during approximately the first three quarters of 1953.

Chemistry.—Weisblat *et al.* (153) have devised an elegant new general synthesis for pteric acid and pteroylglutamic acid. N-tosyl-*p*-aminobenzoate or N-tosyl-*p*-aminobenzoylglutamate is alkylated with an appropriately substituted propylene oxide molecule: the product is oxidised to a ketone, and condensed with 2,4,5-triamino-6-hydroxypyrimidine. Pteric acid or pteroylglutamic acid is then released by a new mild procedure for detosylation (154): it consists in treatment with cold 30 per cent HBr in acetic acid, containing phenol to prevent bromination of the product.

Occurrence, bacterial conversion etc.—Andrews & Schweigert (155) have continued their determinations of the FA¹ and CF¹ activities of natural products. As in earlier studies with bound forms of FA, various methods of enzyme treatment had to be tried to secure the highest release of CF from different materials [see also Chang (156)]. Collins *et al.* (85, 86) find the FA content of colostrum higher than that of normal milk from the cow or goat. Bond (157) has demonstrated conversion by *Lactobacillus casei* of FA to CF while Broquist *et al.* (158) have studied the same reaction with *Streptococcus faecalis*: they find that for maximum yields it requires reducing conditions and a source of "formate," e.g., serine. A strain resistant to amethopterin was more efficient than the parent strain. Similarly Hendlin *et al.* (159) found that both growing and resting cells of several lactobacilli convert FA into CF (largely present in a bound form) and that with resting cells the conversion is proportional to the FA concentration and is enhanced by ascorbic acid. With *Lactobacillus arabinosus* the reaction is partially inhibited by aminopterin, which, however, completely inhibits the conversion from *p*-aminobenzoic acid. It is concluded that the biosynthetic route is:



The synthesis of CF from FA is also efficiently performed by a chick liver homogenate under anaerobic conditions in presence of ascorbic acid [Nichol (160)]. Under aerobic conditions also some CF was formed, and added folinic acid was protected by ascorbic acid from destruction. Glucoascorbic and isoascorbic acids could replace ascorbic acid but cysteine and glutathione were ineffective as found in earlier studies with intact animals. Keresztesy & Silverman (161) have prepared from horse liver a protein fraction that in the presence specifically of L-glutamic acid destroys folinic acid by cleavage of pteridine from the *p*-aminobenzoyl part of the molecule; neither pteroylglutamic acid (PGA) nor N-10 formyl PGA is attacked by this enzyme system; it is inhibited by *p*-aminobenzoic acid. Toennies *et al.* (162) describe a curious labile factor from the dialysates of blood haemolysates; it shows a high activity for *L. casei*, equivalent to some 20 to 100 times the expected

amount of FA. Activity is lost on storage even at 2°C., especially in dilute solutions with high salt concentrations. The activity of the factor for *Streptococcus lactis* is small.

Therapeutic uses.—Das Gupta *et al.* (163) have confirmed that FA is the primary requirement, in most cases of nutritional macrocytic anaemia occurring in India, with vitamin B₁₂ as an additional secondary requirement. Of 22 cases treated first with vitamin B₁₂ although 14 showed improvement, in only 4 was this sustained while macrocytosis persisted in most. PGA¹ given subsequently resulted in further improvement in clinical condition and blood picture, though some cases required treatment with liver extract also. On the other hand, in true pernicious anaemia Meacham & Heinle (73) found that PGA was of no value in addition to vitamin B₁₂ for those few cases showing a persistent mild macrocytosis. This is in line with the results obtained by Clark (164) in FA tolerance tests. A 1 mg. oral dose caused in most individuals a rise in the serum FA from 4 μ g. per ml., or less, to as much as 36 μ g. per ml. in 1 to 4 hr. Normals and pernicious anaemia cases showed a similar wide scatter of results, with a tendency for the pernicious anaemia cases to reach a peak earlier. It is concluded that there is no defect in FA absorption in pernicious anaemia cases but that they may remove FA from the circulation more rapidly, perhaps a result of tissue desaturation. Similar lack of correlation between FA and B₁₂ serum levels is indicated by Mollin & Ross (72). They found no increase in the low serum B₁₂ in pernicious anaemia cases in relapse after PGA by mouth or folinic acid by injection, although the bone marrow became normoblastic. On the other hand, patients with macrocytic anaemia attributable to FA deficiency had normal serum levels of vitamin B₁₂, so that this could be used as a diagnostic test. The two distinct types of macrocytic anaemia in humans may be contrasted with the incidence of this condition in swine: Cartwright *et al.* (96) could only induce it by creating a dual deficiency of both FA and B₁₂ and then could only cure it by supplying both vitamins. The failure of FA to show uniform effectiveness in all cases of "sprue syndrome" may be explained by Manson-Bahr's analysis (165). He considers that three distinct conditions can be distinguished, namely celiac disease of children, steatorrhea, and true tropical sprue, which has a limited geographical incidence and is probably caused by a virus infection.

Girdwood (166, 167) has continued his extensive studies on FA excretion in various diseases. Intestinal malabsorption can sometimes be demonstrated (e.g., in sprue) by failure to excrete FA in the urine after an oral dose, in contrast to normal excretion after subcutaneous administration. Macrocytic anaemia of pregnancy subsequently responding to FA treatment may, however, occur without biochemical evidence for either failure in absorption or tissue depletion. Urinary excretion during 24 hr. of less than 1.5 mg. FA after 5 mg. subcutaneously is indicative of malignant disease, in the absence of other conditions that reduce excretion. Unfortunately, however, excretion is not abnormally low in all malignant cases.

By feeding a diet low in FA with added sulfaguanidine (but no FA antagonist) a deficiency state can be induced in ducklings. This is characterised by reduced growth, macrocytic anaemia and enlarged livers low in glycogen. Administration of vitamin B₁₂ does not counteract these symptoms [Miller *et al.* (168)].

Biochemistry.—The involvement of FA in transmethylation appeared to be well established, and it is something of a shock to have doubt thrown on the hypothesis by Stekol *et al.* (169). They confirm that in rats FA deficiency reduces the incorporation of the methyls of methionine and betaine into tissue choline and creatinine, and that vitamin B₁₂ has no effect on these transmethylations. They find further that pyridoxin deficiency also reduces incorporation of methionine methyl into choline and of the β carbon of serine into both moieties of choline. They then suggest that the decreased transmethylation may be a result of decreased synthesis of appropriate methyl acceptors in pyridoxin and FA deficiency. If this is so, then neither vitamin B₁₂ nor FA, nor pyridoxin may be directly concerned in the transfer of methyl groups.

The effect of FA deficiency on hormonally induced growth has been studied by Silver (170). The deficiency was brought about by giving aminopterin, which had no obvious ill effect on pigeons. Nevertheless, the growth of the crop gland of the pigeons and of the mammary gland of rats was depressed. Since only the extent, but not the type, of growth was found to be affected, it is suggested that FA is concerned with tissue proliferation rather than in mediation of hormonal stimuli. Williams *et al.* (171) find that after rats have been depleted of FA by feeding a deficient diet for five weeks, both the whole liver and the liver mitochondria lose 90 per cent of the initial FA content. In the same period the losses of CF in whole liver and mitochondria are 70 per cent and 30 per cent respectively. In rats on a normal diet aminopterin has no effect on FA storage, but is equivalent to a deficient diet in its effect on storage of CF. The choline oxidase activity of the mitochondria runs parallel to the CF content on these regimes. May *et al.* (172) report that in the monkey, ascorbic acid is not necessary for the conversion of FA to CF. All phases of metabolism of FA compounds are normal in scurvy, and ascorbic acid deficiency is not accompanied by marked deficiencies of FA compounds until signs of scurvy are well advanced. The increased requirements then manifested for FA compounds is regarded as attributable to nonspecific factors.

In *Tetrahymena*, Kidder & Dewy (173) find that conversion of glycine to serine requires a high level of FA and is stimulated by thioctic acid; formate, glyoxalate, and glycolate do not contribute one-carbon fragment to this synthesis. In the rat, on the other hand, the glycine to serine conversion is believed by Stekol *et al.* (174) to be mediated by vitamin B₁₂. Both vitamins appear to be involved in the conversion of glycine to the cysteine and glutamic acid moieties of glutathione [Anderson & Stekol (129)]. Daft *et al.* (175) suggest that the normal metabolism of histidine suffers interference in FA

deficiency. Rats in this condition receiving a diet high in histidine excrete a compound that breaks down on hydrolysis to yield 1 mole each of glutamic acid, formic acid, and ammonia. The same compound has been isolated in crystalline form by Borek & Waelsch (176) from enzymatic degradation of histidine with a cat liver extract. It has been characterised as α -formamidino-glutaric acid.

Totter (177) reports that bone marrow cells can incorporate C^{14} -formate into both serine and nucleic acids. The deoxyribonucleic acids contain four to ten times as much activity as the ribonucleic acids, but the position is reversed when the component purines are examined: the ribonucleic acid purines are two to four times as active as the deoxyribonucleic acid purines. The anomaly is resolved by the finding that most of the radioactivity is concentrated in the 5-methyl group of thymine. Incorporation into this position is suppressed by aminopterin, and the effect is reversed by CF. Buchanan *et al.* (178, 179, 180) find that, on incubation of inosinic acid with C^{14} -formate and pigeon liver extract, activity is found in the 2-position of inosinic acid. This is interpreted as as a result of cleavage to 4-amino-5-imidazolecarboxamide ribotide, followed by resynthesis incorporating formate, with CF activity as the formylating coenzyme. The formyl group in CF is regarded as being exchangeable with radioactive formate, so that CF functions as an acceptor and donor of formyl groups. See also Buchanan & Schulman (179) and reviews by Greenberg (181) and by Shive (182).

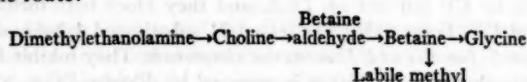
Antagonists.—Additional evidence is available that the toxicity of aminopterin is reversed or prevented specifically by CF. In the rat Sauberlich (183) found FA, ascorbic acid, cortisone, penicillin, or aureomycin all ineffective by injection and only CF effective. By mouth, CF or large doses of FA were effective. Moreover aminopterin reduced the capacity of the liver to perform transmethyations: this reduction could be prevented by dietary FA in large doses as well as by CF. On the other hand Bénard *et al.* (184) found that aminopterin toxicity in the rat could be prevented by simultaneous injections of folic acid in rather large amounts, or, less effectively, by liver extract. Vitamin B_{12} was useless, and all of them were ineffective by mouth. It is pointed out by Jukes (185) that FA antagonists can be divided into two groups. The first group, typified by α -methylpteroylglutamic acid, are reversed by PGA in animal experiments and block the formation but not the utilisation of CF. The second group, typified by 4-amino derivatives of PGA, are reversed by CF but not by PGA, and they block both formation and utilisation of CF. Foley (186) has tested 71 substituted 1,2-dihydro-5-triazines against *S. faecalis* and *Leuconostoc citrovorum*. They inhibit PGA non-competitively, but the inhibition is reversed by dihydro-PGA, N^{10} formyl-PGA or CF: these compounds are believed to interfere with the conversion of FA to CF. A new antagonist, 2-deamino folic acid, is described by Brown (187). Although it is inhibitory to *L. casei* and *S. faecalis*, it does not inhibit Walker rat sarcoma 256, nor does it arrest cell division in chick embryo cells, as does aminopterin.

CHOLINE, BIOTIN, AND *p*-AMINO BENZOIC ACID

Choline.—Cotte & Kahane (188) have used alkaline persulphate in the determination of choline; it has some advantages, but is less specific than alkaline permanganate. Heyndrickx (189) describes a method for the separation of choline and thiamine by chromatography on starch. Riedesel & Hines (190) have studied the absorption of choline from the rat intestine. They find the rate of absorption unaltered by prior incubation of the choline with gut contents or by suppression of bacterial activity with antibiotics. They conclude therefore that choline chloride is absorbed unchanged and that microorganisms play no part in the process. Eddy (191) reports on a particular strain of *Aerobacter aerogenes* that specifically converts choline or acetylcholine to methylamine. The conversion is only brought about by cells in the logarithmic phase and not at all by resting cells. It is inhibited by glucose but not by the presumed reaction product, ethylene glycol.

Polonovski & Bourillon (192) have studied an enzyme system of human and pig bile (probably different from the phosphatase system of bile) that yields phosphate and choline in 1:1 ratio from the phospholipides. Rats on a choline-deficient diet were found by Morgan & Lewis (193) not to develop fatty livers if they were also deprived of pantothenic acid. Adrenal hormone production was depressed in pantothenic acid deficiency and fat metabolism was seriously deranged, so that fat transport to the liver was prevented. The presumed role of choline in preventing fatty livers has been the transport of fatty acids from the liver as plasma phospholipids. However, Artom (194) now suggests that it may instead enhance the oxidation of liver fatty acids. He studied the release of radioactive CO_2 or acetoacetate from C^{14} -stearate on incubation with rat liver. The amounts produced were much greater with rats fed choline than with deficient animals, and highest with the livers of rats recently injected with choline.

Tracer studies by Pilgeram *et al.* (195) and by Soloway & Stetten (196) have added precision to our knowledge of the pathway of choline biosynthesis and degradation in the rat. Ethanolamine is largely excreted as urea and CO_2 , but part is rapidly incorporated into choline and phospholipids. Ethanolamine carbon also appears in several amino acids, including glycine, which according to Pilgeram *et al.* probably arises via choline. In the other study several compounds labelled with C^{14} and N^{15} were used; the results confirm the route:



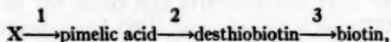
The first reaction is slower than the others. Correspondingly, dimethylethanolamine is far less efficiently converted into glycine than is choline or betaine, so that this is probably the only route to glycine. The ethanolamine residue of choline, including the nitrogen, appears intact in glycine. Surpris-

ingly aminopterin poisoning did not appear significantly to inhibit choline oxidase in the intact rat, because it did not depress the conversion of choline into betaine, although conversion into glycine was decreased slightly.

Doctor *et al.* (197) describe a manometric method suitable for the assay of choline oxidase (CO) in washed suspensions of avian liver. CO is mainly concentrated in the liver mitochondria and could not be extracted therefrom. However, Williams & Sreenivasan (198) have succeeded in obtaining at least part of the enzyme system in solution by treating the mitochondrial preparation with sodium choleate and phosphate buffer, thereby effecting a 13-fold purification. The soluble choline dehydrogenase can utilise as hydrogen acceptor 2,6-dichlorophenolindophenol but not atmospheric oxygen. Flavin-adenine-dinucleotide is involved as coenzyme in choline dehydrogenation [Ebisuzaki & Williams (199)]. By purifying the enzyme from betaine aldehyde dehydrogenase, Strength *et al.* (200) were able to show that diphosphopyridine nucleotide was also required for optimal activity. Swenseid *et al.* (201) have studied the inhibitory effect of ethionine. When fed to rats, it depresses the activity of the liver choline oxidase and sarcosine oxidase. However, by comparing *in vivo* with *in vitro* experiments, they reached the conclusion that some enzymatically produced derivative of ethionine is the effective inhibitor. Feeding ethionine appears to cause reduction in the ability of a series of enzymes to produce one-carbon units. Choline oxidase is apparently not restricted to the animal kingdom: Cromwell & Rennie (202) have discovered in sugar beet roots an enzyme system that converts choline to betaine and is stimulated by cytochrome-*c* like the liver CO¹ system.

Biotin and *p*-aminobenzoic acid.—Assays for biotin, pantothenic acid, and nicotinic acid have been carried by James (203) on many varieties of vegetables, fruits, and nuts. The only kinds showing notable contents of biotin were red and black currants, sunflower seeds, and some nuts; hazel and filbert nuts gave the highest values, namely 14 to 16 μg . biotin per 100 g. Terroine & Rombauts (204) find that biotin does not play any role in the deamination of aspartic acid in the rat, as it does in some microorganisms. The characteristic symptom of biotin deficiency, high ammonia excretion, is regarded as a secondary phenomenon arising from hyperacidity as a result of pyruvic acid accumulation in the blood. Suppression of the high urinary ammonia by sodium compounds does not alter the progress of the deficiency disease in the absence of biotin. The metabolic fate of biotin has been studied by Baxter & Quastel (205) using carboxyl C¹⁴-biotin. This was degraded (under aerobic conditions only) by guinea pig kidney cortex slices and by other tissues. Biological activity was lost, and there was evidence of the removal of one or more two-carbon fragments from the side-chain. The oxidation was inhibited by azide and competitively by some biotin analogues. It is concluded that the enzyme system, biotin oxidase, is probably distinct from that involved in the oxidation of lower fatty acids, because the latter do not inhibit biotin degradation. The biotin requirements of the thermophilic bac-

teria *Bacillus coagulans* and *Bacillus stearothermophilus* have been investigated by Campbell & Williams (206). All strains could utilise oxybiotin or desthiobiotin, but one strain could not use the latter at temperatures above 36°C. Some strains could satisfy their biotin needs with pimelic acid, aspartic acid, or oleic acid. It is concluded that in the biosynthetic reaction chain



some strains are blocked at either step 1 or step 2, but only one is partially blocked at step 3. Wiken & Richard (207) have investigated the growth factor requirements of a large number of new clostridium strains. Apart from a few autotrophs, all required biotin, except that in seven strains biotin could be replaced by *p*-aminobenzoic acid.

The *in vitro* growth of the cartilaginous tibia of embryo chicks in synthetic media has been studied by Kieny (208). Apparently *p*-aminobenzoic acid stimulates growth by rendering the tissues capable of assimilating amino acids. According to Takemori & Kitaoka (209) the growth of rickettsia in tissue culture is inhibited by *p*-aminobenzoic acid, and the effect is reversed by *p*-hydroxybenzoic acid.

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WATER-SOLUBLE VITAMINS, PART II^{1,2}

(PANTOTHENIC ACID, THIAMINE, LIPOIC ACID, RIBOFLAVIN, VITAMIN B₆,
NIACIN, ASCORBIC ACID, AND MISCELLANEOUS FACTORS)

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INTRODUCTION

The year 1953 witnessed the further acceleration of a trend that has been in progress in vitamin research for several years, away from observations on whole animals and toward an examination of vitamin action at the cellular or subcellular level. The latter approach originally furnished information concerning individual enzyme systems, which has been continually expanded into an integrated picture of larger areas of metabolism and physiologic behavior.

The fields of vitaminology and enzymology become identified with each other at the molecular level, and the extension of our knowledge of the finer reaction mechanisms that include these vitamins has enabled biochemists to explain an increasing number of aspects of metabolism in terms of simple chemical reactions. Especially valuable, for example, have been the identification of "active acetate" as acetyl coenzyme A, and the discovery of the importance of other acyl coenzyme A compounds in fat metabolism; the studies on interaction between coenzyme A and thiamine and lipoic acid containing systems are clarifying to a considerable extent the important details of the metabolism of two-carbon compounds. The finding that metals occur as integral units of flavoproteins, and new information on the behavior of ascorbic acid, add to the assurance that the current emphasis on enzymology in vitamin research will be maintained.

¹ This review deals with the vitamins connected with C₁ metabolism (pantothenic acid, thiamine, and lipoic acid); with the electron transport vitamins, riboflavin, and niacin; the B₆ group; ascorbic acid; and new growth factors. Inositol is omitted because of the relatively few papers that were devoted to it during the past year.

The literature survey was concluded with journals available to the authors to December, 1953.

² The following abbreviations are used: Acetyl LBF for acetyl pantetheine; ADP for adenosinediphosphate; AMP for adenosine-5'-phosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; TPP for thiamine pyrophosphate (diphosphothiamine); FAD for flavin-adenine-dinucleotide; FMN for flavin mononucleotide; G-3-P for glyceraldehyde-3-phosphate; LBF for *Lactobacillus bulgaricus* factor = pantetheine; LBF- γ -phosphate for pantetheine-4'-phosphate; LTPP for lipothiamide pyrophosphate; NMN for N'-methyl nicotinamide; PA for pantothenic acid; PAB for p-aminobenzoic acid; PAC for pantothenic acid conjugate; P-P for inorganic pyrophosphate.

This emphasis, which will also be felt in the present review, should not be interpreted to detract from the significance of many nutrition type experiments. These continue to be necessary, not only for themselves but also to provide a background against which subcellular studies may be evaluated. They have been included in this discussion wherever possible, within the space limitations imposed.

PANTOTHENIC ACID

Information has accumulated during the past year which consolidates the position of coenzyme A (CoA³) in the formation of "active acetate," and also gives further evidence of the versatility of this coenzyme as a biocatalyst. Since several of its functions are considered in detail in the chapter on carbohydrate metabolism, it will suffice here to record that CoA has been shown in the laboratories of Green, Lynen, Ochoa, and Stadtman to be a key component in systems for fatty acid breakdown, through the formation of acyl and β -ketoacyl CoA derivatives (1 to 5), probably at each step in the "cycle" (3) whereby the fatty acids are oxidized into C₂ units (acetyl CoA) (5). Moreover, the reactions appear to be reversible in purified enzyme preparations (5, 6). The latter studies thus confirm at the molecular level the observations (7, 8) that CoA catalyzes the formation of longer chain fatty acids (as well as sterols) from acetate and open to view the extended series of degradations that occur during the burning of fatty acid molecules. Related observations (9, 10) indicate that α -glycerol phosphate is esterified with fatty acyl CoA derivatives, and thus the biosynthesis of phospholipids and neutral fats may also be foreshadowed through the use of this coenzyme. Other reactions that appear to be CoA-dependent are the enzymatic decarboxylation of malonate (11) and succinate (12, 13) in microorganisms, the furoylation of glycine in liver and kidney preparations (14), and probably the acylation of glycine in *Clostridium kluyveri* (14a). Since neither of the latter decarboxylations also involves a carbonyl group in the substrate (in contrast to pyruvate, α -ketoglutarate, or β -keto acids), this may be viewed as a new type of CoA-dependent cleavage (possibly through malonyl or succinyl phosphate), to yield acetate and propionate, respectively. The co-dependence of succinate decarboxylation upon thiamine pyrophosphate (TPP²) and the simultaneous formation of CO₂ in both reactions, may suggest an interaction between the CoA-systems and those involved in production of the active C₁ fragment.

The importance of acyl CoA derivatives has prompted several workers to investigate methods for their preparation in highly purified form. Numerous enzymatic syntheses have been described (1, 15, 16). Among the nonenzymatic methods, thioacetic acid (17), S-acetylthiophenol (18), thiopalmitic acid (10), and succinic (2), crotonic (19), and benzoic (20) anhydrides have been used as acylating agents. Acetyl pantetheine (Acetyl LBF²) has been made from LBF² with either acetylthiophenol (18) or acetyl chloride (21), or from pantothenic ethyl imide with thioacetic acid (22). High (up to 90 per

cent) purity preparations of CoA for these reactions can be readily isolated by adsorption and elution from charcoal (23), followed by chromatography on charcoal (23, 24) and Dowex 50 or Dowex 1, with or without the intermediate co-precipitation of CoA with glutathione as a copper salt.

Although the structure of CoA has not yet been completely confirmed by synthesis, it seems reasonably clear that the configuration given in a previous review (25) is essentially correct. Hydrolysis of the third phosphate group (a monophosphate) indicates (26) that the linkage corresponds to that in adenylic acid *b*; this may suggest attachment at carbon 3' of the adenylic residue. Other data relating to degradation of CoA and enzymatic resynthesis, which generally confirm the structure of the coenzyme, have been effectively reviewed elsewhere (27 to 30). The conversion of LBF to CoA in liver tissue was pictured as proceeding via (a) a phosphorylation (with ATP²) to produce pantetheine-4'-phosphate (LBF- γ -phosphate²) (30, 31); (b) condensation of the latter with ATP to give "dephospho CoA"; (c) phosphorylation of this product with ATP to yield CoA (32). These reactions were catalyzed by soluble, nonparticulate enzyme fractions. Yields of 80 to 90 per cent were reported for the conversion of LBF- γ -phosphate to CoA (29).

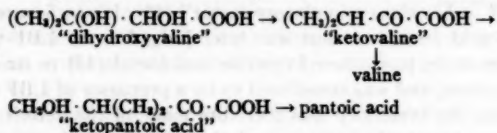
The isolation of LBF of about 50 per cent purity has been described, from culture filtrates of *Ashbya gossypii* (33), and methods for the synthesis of this compound in pure form have been developed in several laboratories. Four of these procedures (34 to 37) involved the condensation of the sulfur-containing moiety with β -alanine or a derivative to form " β -aletheine" (35), followed by coupling with pantolactone. A fifth method (22) employed the formation of pantothenic ethylene imide, followed by S-acylation with thio- or phenylthioacetic acid, and hydrolysis of the mercaptoester. Good yields were generally claimed.

The question naturally arises concerning the possible biological activity *per se* of the various moieties and sub-units of CoA. Several of them are active presumably as precursors of CoA. An organism that responds especially well to these forms is *Acetobacter suboxydans*, which was early shown to be more responsive to CoA and PAC² (38, 39, 40) than to free pantothenic acid (PA²). Likewise, the pyrophosphatase-split product from CoA was active, and was called the "*A. suboxydans* stimulatory factor" (41). It was first regarded as a pantothenic acid phosphate but was later judged to be LBF- γ -phosphate (42). More recently, pantothenyl cysteine was found (43) to strongly stimulate this organism, and was considered to be a precursor of LBF and CoA in *A. suboxydans*. An inventory was therefore made of the activity of various PA derivatives for the bacterium, and the following order of effectiveness was established (44, 45): LBF- γ -phosphate = CoA > pantothenyl cysteine = LBF (approx.) > PA = pantoic acid > LBF diphosphate \geq PA- α - or - γ -phosphate (\rightarrow 0), with CoA and LBF- γ -phosphate about 10 to 20 times as active as CoA. It is evident from these results that growth stimulation in *A. suboxydans* is not the property of a single compound in the PA-CoA family. Of interest was the fact that the sulfur-containing structures were active princi-

pally, if not exclusively, in the reduced state. Apparently this organism, despite its facility for converting pantoic acid through several intermediates into CoA, lacks the ability to reduce the S—S bond in these compounds once they are formed.

The foregoing series of studies do not support the idea that PA derivatives other than CoA may possess intrinsic enzymatic activity. The latter view might be held, from analogy with flavin- and pyridinenucleotides, and from the observation (46) that rat growth assay values for PA were higher than microbiological values in certain materials, under conditions where CoA and other known PA conjugates were believed to be completely converted to PA for the microbial tests. The higher rat assay values may have a partial explanation in the finding (43) that avian liver enzyme preparations which cleave CoA to PA are only feebly active upon pantothenylcystine. If the latter compound were active for the rat, its presence in natural materials could contribute to the total apparent PA content, and indeed the microbial values would then be spuriously low on account of incomplete liberation of PA by the enzyme treatment. The distribution of pantothenyl cyst(e)ine in natural materials requires additional study, and a further inventory of PA derivatives is needed, against the possibility that minor, yet possibly important relatives of CoA may have thus far been obscured from view.

The biosynthesis of PA has also been examined. It is reportedly increased in streptomycin-resistant strains of *Escherichia coli* (47). The reason is unknown but the experiments recall the report (48) that an alternate scheme may exist in some organisms for the condensation of pyruvate and oxalacetate, which is affected by streptomycin. Possibly the CoA requirements of such a condensation (and the succeeding degradations) are different from those encountered in the traditional Krebs cycle. The coupling of β -alanine to pantoate in *E. coli* has been shown to be mediated by ATP, with cleavage of the latter to form AMP³ and P-P³ (49). ADP³ could not substitute for ATP as an energy source. Preliminary to this coupling, pantoate was thought on the basis of studies with *E. coli* mutants (50) to be formed from "dihydroxyvaline" or "ketovaline," which had also been viewed as precursors of valine (51). The proposed reaction sequence is shown, as follows:



This sequence differs from earlier suggestions regarding pantoate synthesis, in that neither valine (52) nor the α -amino analogue of pantoic acid [pantoinine (53)] is regarded as a direct precursor. However, "ketopantoic acid," in contrast to pantonine, has been shown in two laboratories (51, 54) to be an effective growth promoter in a pantoicless mutant and also to be a potent reversing agent of salicylate inhibition. Evidence for the steps preceding the

formation of ketopantoic acid was obtained in the manner common to mutant studies: accumulation of intermediates in blocked mutants and growth of the latter with supposed products of these intermediates. Although such experiments when taken alone may not reveal the major normal pathways of metabolism, nevertheless the scheme offered appears to fit the available data and furnishes a pattern for further examination of these interconversions.

The formation of ketopantoate in the foregoing outline involves a hydroxymethylation; this may suggest the participation of the folic acid-citrovorum factor group. Of possible special interest, then, are the continuing studies on *Bacterium linens* (55, 56), in which the biosynthesis of PA was mediated by *p*-aminobenzoic acid (PAB³) (although only slightly by folic or folinic acids). These studies may thus also point to a possible function for PAB apart from its contribution to the folic acid molecule. In addition, the unique feature was presented that PA and PAB were alternately required for growth; in the presence of either vitamin, the other was synthesized. Growth inhibition by sulfanilamide could be reversed not only by PAB but also by PA, pantoic acid, or biotin. The interrelationships that exist here are unclear, but they recall related observations in rats (57) and *E. coli* (58). It seems evident that PAB functions in the synthesis of PA, and possible that CoA or biotin or both may be involved in the formation of PAB or other aromatic compounds. Experiments with appropriate cell-free systems should aid materially in explaining these interesting relationships.

The interactions between PA and other vitamins or hormones are even less understood. Much attention has been paid to the influence of PA, as well as ascorbic acid,³ on adrenal function, and progress in this field has been reviewed elsewhere (30, 59, 60). In passing, it should be emphasized that a close relationship exists between PA (presumably as CoA) content of the tissues and adrenal cortex function. Although many of the changes are complex, perhaps as a result of participation of the pituitary, it would appear that most of the adrenal dependence upon PA has its basis in the catalysis of steroid synthesis, particularly cholesterol, by CoA. Some current articles in this field (61, 62) reporting the response of normal and PA-deficient rats to stress, fit reasonably into this pattern, although in two studies (62, 63) it was suggested that some cortisone-like hormone or hormones may still be produced in the PA-deficient rat. The complex character of this deficiency is exhibited by the fact that, despite many evidences of improvement in adrenal function by administration of adrenal cortex hormones to PA-deficient rats, neither cortisone, ACTH, thyroxine, growth hormone, nor anterior pituitary extract was able to improve their resistance to a cold environment (64). Thyroxine was suggested as a possible regulator of the CoA level in the tissues (65), on the basis of an observed increase in CoA after administration of the hormone to thyroidectomized animals.

³ For a discussion of ascorbic acid-adrenal relationships, cf. section on ascorbic acid.

A colorimetric assay has been developed for PA (66) which depends upon acid hydrolysis and coupling of the liberated pantoic acid with 2,7-naphthalenediol. The method is rapid, and good precision and agreement with microbiological values are claimed, among the high potency vitamin mixtures tested. The method was apparently not intended for use with most natural materials, and indeed it seems probable that many interfering compounds normally present would invalidate the assay.

Alloxan inactivation of CoA (nonenzymatic) has been reported (67), with the suggestion that the diabetogenic effect of alloxan may in part be attributable to its reactivity with the sulfhydryl group of CoA in the beta cells of the pancreas.

In an effort to obtain a better understanding of the chemistry of the thioester bond, numerous model compounds and reactions have been examined, including the reaction between amines and thiocarboxylic acids (68), and the cleavage of various thioesters by hydrolysis or aminolysis (69, 70). Substituent groups in the compounds were varied considerably. The thioacid or thioester groups were all highly reactive, with aminolysis (including reaction with hydroxylamine) being more rapid than alkaline hydrolysis, and this in turn more than acid hydrolysis. The competition among these reactions in aqueous solution thus greatly favored the hydroxylamine reaction as has been regularly observed with acetyl CoA or acetyl LBF. The reaction rates for ethylthioacetate and β -acetaminoethyl thioacetate were similar (70)' and the presence of an amide group on the β -carbon therefore exerted only slight influence on the reactivity. It would appear that the activity of the S-acyl bond in CoA derivatives may not differ greatly from those in the models studied, as would also be expected from the use of similar model compounds (S-acyl-N-acetylthioethanolamines) by Lynen in fatty acid oxidation studies (3, 71). CoA preparations have been found stable for 2 years at room temperature in the dry state, and several months in neutral aqueous solution. In the presence of 0.5 N H_2SO_4 , 20 to 30 per cent was destroyed in a few minutes (72). Replacement of acyl by aryl in LBF derivatives (S-phenyl LBF) produced a growth antagonist for *Lactobacillus helveticus* (73).

Virtually all of the known biochemistry of CoA at present centers about the —SH group, because of its importance and the novelty of many of its functions. Although, as pointed out by Lipmann (74), there may be a tendency to overemphasize this group, it has so far yielded extremely valuable information. A possible further property of CoA that has scarcely been examined is a potential role in phosphate energy transfer (75), where a suggested intermediate compound is an enzyme-phosphoryl~S-CoA. This intriguing observation deserves major attention in future research.

THIAMINE

Crude allithiamine was first prepared (76) by heating thiamine at pH 8 with an alcoholic extract of garlic (*Allium sativum* L. var. *japonicum* Kitamura). The product was negative to the thiochrome test but positive after

treatment with cysteine. The pure compound was later isolated (77) and shown to have the structure given in Figure 1, formula I. The homologues with other alkyls, such as methyl, propyl, were also formed during the reaction at pH 8 and 50 to 60°C., especially with other species of *Allium* (78). Allithiamine and its homologues could be reduced by cysteine into a thiol form of thiamine (V), as shown in Figure 1.

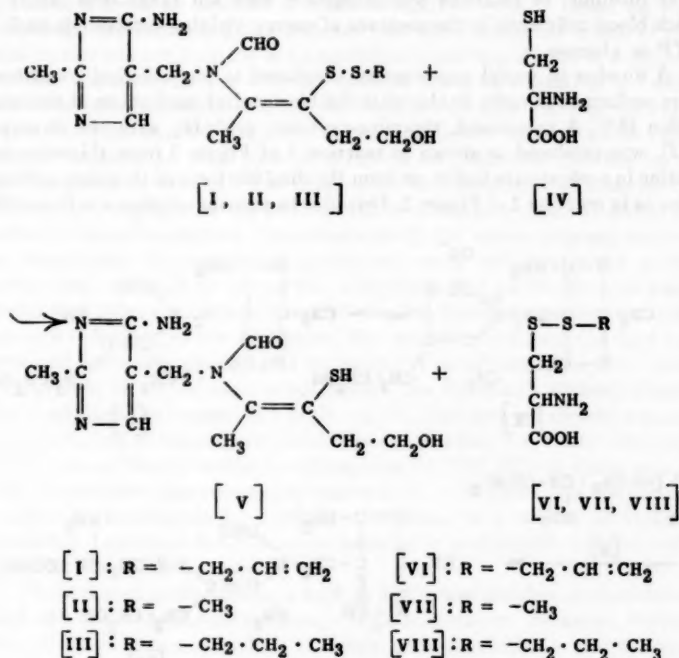
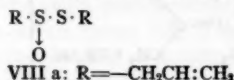


FIG. 1. Reduction of allithiamine and homologues by cysteine.

The byproduct S-allyl mercaptocysteine from the allithiamine reaction had been obtained previously (79) from alliin (VIII a) and cysteine.



The formation of allithiamine (I) could thus be explained by the reaction between the thiol form of thiamine and alliin (VIII a). The latter was found earlier as an enzymatic decomposition product of alliin (80). Since other homologues were also produced, it is likely that the plants contain other alliin-like compounds.

Since allithiamine was freely convertible to thiamine by cysteine, then it should possess vitamin activity. This was found to be true for rats on a thiamine-free diet (76, 81). It was also active for many fungi and was believed to be converted to thiamine (81). Because allithiamine and its homologues were quite soluble in many organic solvents, their uptake by blood cells *in vitro* could be readily followed (82). Under the same conditions, thiamine, thiamine disulfide, or thiamine pyrophosphate were not taken into rabbit or chick blood cells even in the presence of energy yielding compounds such as ATP or glucose.

A number of model experiments, simulated to physiological conditions, were performed in order to elucidate the biochemical mechanism of thiamine action (83). A compound, thiamine-cysteine, probably with the structure (XI), was produced as shown in reaction 1 of Figure 2 from thiamine and cysteine in a phosphate buffer, or from the disulfide form of thiamine and cysteine as in reaction 2 of Figure 2. Thiamine in aqueous solution was in equilib-

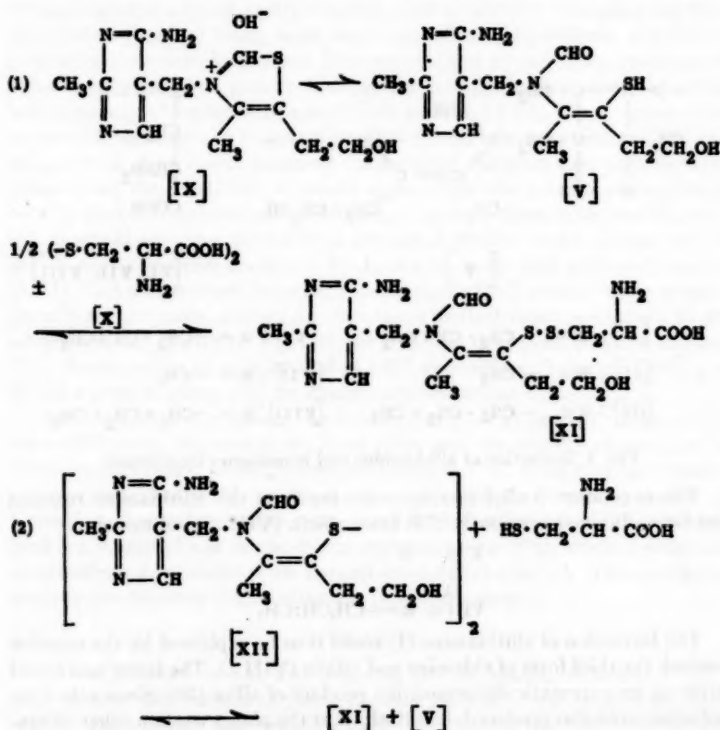


FIG. 2. Formation of thiamine-cysteine.

rium between the ammonium (IX) and thiol forms (V). The equilibrium of IX and V is not a simple reaction, however, for there is an internal redox reaction: the states of nitrogen, sulfur and carbon are altered. Lack of physical data prevents a prediction of the relative amounts of each form present. On the other hand, in the cysteine-cystine containing system, the over-all



equilibrium could be depicted as $(XI) \rightleftharpoons (XII) \rightleftharpoons (XI)$. Based on these reasonings, the Japanese group believed that the biochemical action of thiamine *in vivo* was attributable to an equilibrium reaction similar to reaction 1, and that the catalytic action of the vitamin would be a result of the oxidation of certain substrates affected by thiamine-cysteine (XI) or its homologues. They felt that the vitamin action was not attributable to a redox equilibrium between the thiol form of thiamine and thiamine disulfide as had been proposed earlier (84). The observation is interesting and deserves further study. Judging from the known actions of thiamine (such as simple and oxidative decarboxylations, transketolations), the above proposal seems to be prematurely drawn. In living systems, many biologically active thiol compounds, especially those recently discovered, such as thioctic acid, pantotheine, and coenzyme A, would complicate the above relationships and could not easily conform to the suggestion. For example, it is believed that thiamine and lipoic acid are required for oxidative decarboxylation. The precise arrangement of the cofactors is not known (see lipoic acid section). Regardless of whether the suggestion (85) is correct, that two coenzymes are separately attached to animal pyruvic oxidase, or whether they exist in the conjugated form of "lipothiamide pyrophosphate," LTPP, (86), it seems cumbersome to speculate that yet another molecule, such as cysteine may be attached to the same apoenzyme for oxidative function. As a matter of fact, the "oxidative" aspect of the α -ketoacid oxidases is attributable to other cofactors than the thiamine moiety.

The action of many cofactors, such as flavin- and pyridine nucleotides and CoA, can now be explained in terms of organic chemistry. However, the function of thiamine pyrophosphate in simple decarboxylation is still unknown. Possibly the CO_2 group of the substrate is first transferred to thiamine pyrophosphate; if so, the form of the carboxylated cofactor remains to be elucidated.

Bracken thiaminase has been resolved into two components, viz., a thermostable dialyzable cofactor and a thermolabile apoenzyme (87). The apoenzyme was purified to a certain extent (88). The thermostable portion was found to exist in many other natural materials, such as yeast, liver, urine, grass. The most active concentrates contained appreciable amounts of several amino acids. DL-proline and L-hydroxyproline were active *in vitro* as the cofactor, while many related amino acids showed less activity. Bracken thiaminase, like the other thiaminase systems present in carp, clams, and *Bacillus thiaminolyticus* (89, 90), could use many metasubstituted aromatic amines as cofactor for the action.

The apoenzyme of carp thiaminase was also purified (91) by fractionation

with ammonium sulfate. It was presumably not identical with the bracken apoenzyme (92). A cofactor was likewise required for its action. The thiaminase actions of both carp and bracken could thus be defined as in Figure 3. The products were the thiazole moiety of the vitamin and a conjugate which contained the pyrimidine portion of thiamine, although the products of bracken thiaminase were not definitely identified. Harris, in a study of biosynthesis of

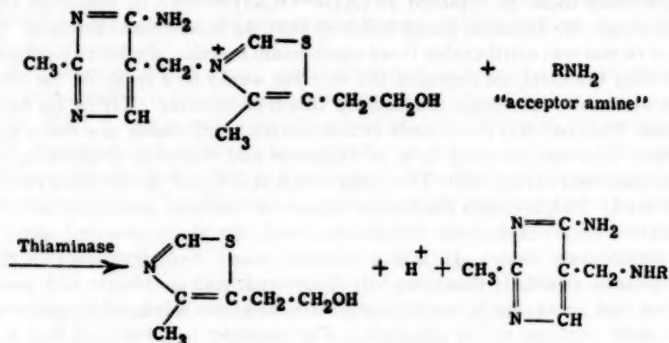
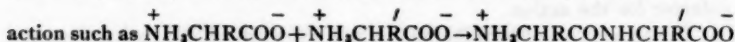


FIG. 3. Mode of thiaminase action.

thiamine in *Neurospora*, found that the pyrimidine moiety preferentially condensed with some unidentified precursor of thiazole to form a thiamine-like intermediate which was secondarily transformed to the vitamin (93). The strains which failed to carry out the latter reaction accumulated this intermediate, the nature of which was not determined. From the experiments described, one possibility may be pyrimidine-amine. Fujita and co-workers (94) believed, however, that the thiaminase reaction was simply a base exchange. From this observation as well as his own, Woolley (91) proposed a very interesting hypothesis to supplement the phosphorus-involving mechanism in biosynthesis. According to this theory, the energy for many biosynthetic reactions which apparently do not involve phosphate could be derived from the reduction of quaternary bases, such as ammonium or sulfonium compounds. He estimated that the free energy change in the thiaminase reaction was about 7000 to 8000 cal. per. mole. Although this figure was only approximate, it was sufficiently high to drive many endergonic reactions. This class of reaction has received less attention, so that little information has accumulated.

Future work will likely be shifted in this direction, to evaluate the $\text{C}\sim\text{N}^+$ bond and other "onium" compounds, not only in thiamine but also in other related structures. It is also interesting to speculate whether any of the routes of protein synthesis, which involves the conversion of ammonium to trivalent nitrogen, may have some connection with the foregoing hypothesis. A reaction such as



should be thermodynamically feasible under physiological conditions without additional driving force from other "energy-rich" compounds.

Horecker *et al.* (95, 96) observed that enzyme preparations from spinach or rat liver catalyzed the conversion of pentose phosphate to sedoheptulose phosphate and glyceraldehyde-3-phosphate (G-3-P₃). This conversion required thiamine pyrophosphate. The preparation from spinach contained about 0.8 mole of thiamine pyrophosphate per mole (10,000 gm.) of protein. In contrast to pyruvic carboxylase, the resolution of the spinach enzyme could be achieved by treatment at acid pH in the presence of 45 per cent saturated ammonium sulfate. This behavior suggests that the acid group of thiamine pyrophosphate is attached to the protein, whereas in carboxylase, the stronger attachment should be through the basic group [as also shown experimentally (97)]. The apoenzyme also catalyzed the reaction of L-erythrulose and D-G-3-P to form a mixture of pentose phosphate and heptulose phosphate. Neither product was formed in the absence of thiamine pyrophosphate. Racker *et al.* (98) isolated a crystalline enzyme from bakers' yeast which resembled the spinach enzyme in pentose metabolism. It catalyzed the conversion of ribulose-5-phosphate to D-G-3-P, as well as the decarboxylation of hydroxypyruvate. Both reactions required acceptor aldehyde, such as ribose-5-phosphate, glyceraldehyde, or glycolaldehyde. The yeast enzyme was also thiamine pyrophosphate dependent. This enzyme was named transketolase (98). The discoveries of the new roles of thiamine in oxidative decarboxylation and in transketolation crystallize the importance of this vitamin in animal as well as microbial nutrition. It has seemed surprising that these organisms required thiamine, even though they did not possess a major simple decarboxylative pathway for pyruvate, such as found in yeasts and higher plants.

A relationship between thiamine and pyridoxine has been recognized for some time in certain strains of yeast and other microorganisms. Various theories have been proposed. A thorough investigation has recently been made (99, 100) on one strain of a top brewer's yeast which requires an exogenous supply of either vitamin for the attainment of rapid growth, and of both vitamins for maximum growth. The decarboxylation rate was also slower in the thiamine deficient yeast, but this could be corrected by the addition of thiamine pyrophosphate. The requirement was attributable to its inability to synthesize pyrimidine at a rate needed to support optimum growth and, to a lesser extent, by its retarded rate of synthesis of thiazole. Pyrimidine was found to produce a sparing effect upon thiamine equivalent to that of pyridoxine, and pyridoxine was found to stimulate the rate of synthesis of thiazole. The data were interpreted by postulating a reversible interrelationship between thiamine and pyridoxine with pyrimidine as the key intermediate.

Pyridoxineless mutants of *Neurospora crassa* and *N. sitophila* (101) grew better when thiamine was also added to the culture medium. The stimulatory action of thiamine in this case was found to be a result of competitive inhibition by thiamine of the destruction of pyridoxine. Pyridoxine, in turn, interfered with thiamine synthesis, but sufficient endogenous thiamine was present to satisfy the normal growth requirements. In *Lactobacillus fermenti*

36, it was found that ascorbic acid has thiamine sparing action (102). The addition of both ascorbic acid and thiamine gave a greater growth response than either one alone. Pyrithiamine inhibited the utilization of ascorbic acid for growth in a thiamine-free medium. The microorganism could synthesize thiamine when ascorbic acid was present. Therefore the thiamine sparing action was thought (102) not to be attributable merely to the reducing power of ascorbic acid. In the use of *L. fermenti* for thiamine determination in natural materials, this effect should be considered.

After the intraperitoneal injection into rats of thiamine labeled with C^{14} at position 2 of the thiazole ring, about 66 per cent of the radioactivity was excreted in the urine (103). Five thiamine-containing compounds, including cocarboxylase, were observed. Another appeared to be a 6-thioctic acid conjugate. The experiment employed rather large dosages of thiamine, and applicability to normal metabolism of the findings from such an experiment may be regarded with reservation. This is also true in an experiment with S^{34} labeled thiamine (104), where radioactive sulfur was found in various fractions of sulfur in the urine, after administering the labeled vitamin to thiamine-deficient rats. Administration of oxythiamine reduced the excretion of radioactive inorganic sulfate and increased the neutral sulfur fraction.

Intravenous injection of a large dose of the pyrimidine moiety of thiamine produced a transient but significant increase in the volume rate of gastric secretion but usually no effect on the acidity of the juice (105). The effect of thiamine to raise blood pressure and increase heart rate was clarified as attributable to the acidity of the vitamin rather than its specific action (106).

Thiamine is unstable in usual laboratory rations and becomes more so in the presence of fat. It can be stabilized by addition of reducing compounds, such as ascorbic acid, cysteine, hydroquinone, by sulfasuxidine or glycerol, or by storing in a dry state or under an inert atmosphere (107). A modified method for thiamine determination was claimed to be superior to previous ones, by preliminary ferricyanide oxidation in acid to remove an interfering nicotinamide metabolite, F_2 . Neopyrithiamine has been shown to possess antithiamine activity in the pigeon (108). Reviews on metabolic functions of thiamine (86) and on antithiamine (90) have appeared.

LIPIC ACID (6-THIOCTIC ACID)

Because of the intimate role that lipoic acid has been found to play in pyruvate oxidation, an extended discussion of this compound is given in the chapter on carbohydrate metabolism.

Lipoic acid has perhaps not earned the title "vitamin" in the classical sense, since it has not yet been shown to be required in animal diets (109). Such an objection has little real value, however, since this compound certainly qualifies as an essential biocatalyst in many systems. As has been pointed out (110), enzymatic behavior may under some conditions be fully as valid a criterion as growth in measuring the prosperity of an organism. Our knowledge of the chemistry and biochemistry of lipoic acid have proceeded at a

rate seldom equalled in vitamin research. Since last year's review (111), in which the structure of the factor (protogen; acetate replacement factor; pyruvate oxidation factor) was described as 6-thioctic acid, this compound has been shown by Gunsalus, Reed and others to occupy a vital role as a primary codehydrogenase in microbial oxidation of pyruvate and α -ketoglutarate [(86, 112); see also other reviews on this subject (113, 114)]. A second role for lipoic acid appears as a transfer agent of acyl groups to CoA, whereby acetyl CoA is formed; whereas a third, and perhaps the most interesting function is as an energy transfer agent through the possession by lipoic acid of an S~ bond (86, 112, 114).

The conversion of oxidative energy to S-acyl "high" energy evidently occurs at a relatively low potential; the redox potential of lipothiamide pyrophosphate (86, 111) has been calculated at approximately -0.42 volt at pH 7.0 (86). Such a low value places this cofactor very close to hydrogen on the redox scale and adds credibility to the intriguing suggestion of Calvin and associates (115, 116, 117) that lipoic acid may operate very early in the acceptor chain of hydrogen from water during photosynthesis, and that the thiotic disulfide ring may participate in the primary quantum conversion act of

photosynthesis. The reaction is viewed as chlorophyll (activated) + S~S \rightarrow chlorophyll (ground state) + S~S whereby the free thiyl radicals are produced by electronic excitation and can serve as the hydrogen acceptor system. A similar postulate has been made for the key reaction in the visual process [Strauss (118)], since Wald & Brown (119) had found that the bleaching of rhodopsin is accompanied by the liberation of two —SH groups per molecule of retinene released. Conversely, the retinene + opsin \rightarrow rhodopsin conversion required —SH group participation. The liberation of these —SH groups during bleaching has been considered essential in the initiation of the electrical processes of vision (119). These hypotheses have not yet been supported by direct experiment, although indirect evidence that is in line with Calvin's suggestions has been described (117). Both schemes appear generally plausible, and if they can be validated they will go far toward improving our understanding of both of these important photochemical changes.

The structure of the functional form of lipoic acid is of major interest. Jansen *et al.* (85) have suggested that the thiamine coenzyme may be separate from lipoic acid or other cofactor at the protein surface, whereas Reed & DeBusk (86, 111) consider the thiamine-lipoic conjugate, "lipothiamide pyrophosphate" (LTPP²) (see formula XIII) to be the active codehydrogenase. Despite the differing conclusions, the experimental data in these papers may not be in serious conflict. The identity of LTPP has not yet been rigorously shown, and until it is more firmly established the structure proposed may be regarded with some reservation. Thus, as pointed out elsewhere (114), with the sulfur atoms essential for catalytic activity, only the pyrophosphate group remains available for attachment to the apoenzyme. Such a linkage

sis of riboflavin has been examined (125, 126). With C^{14} -formate, the radioactivity was found almost completely in carbon position 2, but bicarbonate gave predominant labeling at carbon 4. Other substrates such as $C^{14}H_3COOH$, $CH_3C^{14}OOH$, $NH_2C^{14}H_2COOH$, $NH_2CH_2C^{14}OOH$ and uniformly labeled glucose led to a wider distribution of the isotopes. Ring C of the vitamin possessed a labeling pattern similar to that of the pyrimidine portion of purines. No mechanism was offered for the formation of the aromatic ring, but the side chain was thought to arise by a C_2+C_3 condensation.

The maximum formation of riboflavin by *Eremothecium ashbyii* was found to require two unidentified factors (127). Factor A was associated with oleic acid, some of its derivatives, and yeast extract. Factor B occurred in gliadin, caseins, and "vitamin-free" casein. These factors were apparently not directly linked with the growth of the microorganism. The synthesis of riboflavin in *Aerobacter aerogenes* was believed to be inhibited by aureomycin and terramycin (128), while aureomycin competitively inhibited the utilization of riboflavin by a riboflavin mutant of *Bacillus subtilis* and other bacteria (128).

Bioluminescence has been recognized as an enzymatic reaction, but the mechanism has not been understood until recently. Cell-free and particle-free extracts of *Achromobacter fischeri* were prepared (129, 130) which showed continued luminescence. Diphosphopyridine nucleotide (DPN^2) was required for the light-emitting process. Long chain (C_7-C_{18}) fatty aldehydes were also stimulatory (130, 131). Based on these observations, it was suggested that DPN was either closely linked to the light-emitting system or was identical with bacterial luciferin. However, other workers (132) found that a flavin was essential for reduced DPN to function in initiating light emission in purified bacterial extracts. Flavin mononucleotide (FMN^2) was the only derivative that could function in this capacity. In addition to FMN and reduced diphosphopyridine nucleotide ($DPNH^2$), at least one other factor which was presumably identical with bacterial luciferin was required for light production. This finding was in line with the fact (133) that certain dim strains of luminous bacteria would luminesce brightly when grown in the presence of riboflavin.

Riboflavin-5'-phosphate, flavin-adenine-dinucleotide (FAD^2) and a new FAD with the structure of a 4',5'-cyclic phosphate (134) are presumably the biochemically active forms of riboflavin. Four FAD enzymes were found also to contain metals. They are iron in DPN -cytochrome reductase (135); molybdenum in xanthine oxidase (136, 137) and in nitrate reductase (138); and copper in acyl coenzyme A dehydrogenase (139). In this connection, it is interesting to observe the iron requirement in the phosphoroclastic degradation of pyruvate by *Clostridium butyricum* *in vitro*. This system required co-carboxylase, coenzyme A, and Fe^{++} (140). The latter could not be replaced by many other common bivalent cations of which Co^{++} , however, showed very slight activity. Although the metals in these oxidases are nondialyzable, they evidently exist in a form which is able to accept and give up electrons easily. The mechanism of oxidation-reduction (135, 139) is believed to con-

sist of an intramolecular electron transport from FAD to the metal. The reduced metal is then oxidized by the subsequent enzyme. Two moles of FAD and one atom of molybdenum were found per mole of xanthine oxidase (136). If both FAD groups are initially reduced, then the valence change in molybdenum must be at least 4 in order to permit their reoxidation. However, such a change of valence would probably involve amphoterism in molybdenum unless peroxide formation is assumed. Alternately, intermolecular oxidation is complete. These restrictions need not apply to DPN-cytochrome reductase, where the FE:FAD ratio is 4. Whether the attachment of metals in the flavoprotein enzymes is similar to that of iron in heme proteins is not known. It seems likely that the metal may be coordinated with FAD, or be attached to the protein adjacent to the FAD group, similarly to the proposed metal-protein attachments in certain proteolytic enzymes (141). In this way the metal could be dissociated, as well as easily reactivated.

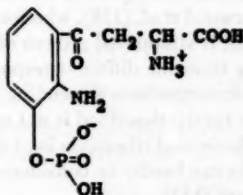
Quinacrine hydrochloride (Atabrine) at 0.04 to 0.08 per cent of the diet exerted a sparing action on riboflavin in rats (142). The drug increased the urinary excretion of riboflavin and enhanced the growth of young rats on rations with suboptimal amounts of the vitamin. The liver riboflavin content was not affected by quinacrine. Its riboflavin-sparing action was explained as possibly attributable either to inhibition of the metabolic destruction of riboflavin or to the structural similarity of the two substances, so that the drug might partially replace the vitamin in flavin-containing enzymes. Some other structural analogues such as phenazines, were found to function in the hexosemonophosphate system. However, it had been previously reported (143, 144) that quinacrine competitively antagonized FAD and FMN. It would be of interest to determine whether quinacrine can attach to an apoenzyme and thus function as a hydrogen transport catalyst. Meanwhile, although stimulation of intestinal synthesis of riboflavin was ruled out in these experiments, it appears possible that quinacrine may function to repress intestinal organisms that normally compete with the host for dietary flavin, and hence improve the growth rate of the animal. This type of explanation is widely used to account for the growth-promoting effects of antibiotics.

Rabbits receiving continuous injections of sodium acetoacetate and β -hydroxybutyrate (145) over long periods exhibited riboflavin and niacin deficiencies. The concentrations of both vitamins in the blood were lowered. This fact was possibly a result of the extra demand upon flavo- and pyridinoproteins, which in turn depreciated these vitamins for other metabolic functions. However, this experiment does not necessarily indicate that diabetics would become deficient in these vitamins, since these keto acids are not completely dissimilated.

Tryptophan is believed to be a precursor of nicotinic acid in many phyla, although the biosynthetic pathway has not been completely explored. In the rat, riboflavin and pyridoxin are definitely involved in the conversion. Kynureninase, which has been shown to be pyridoxal phosphate-dependent (146), catalyzes the conversion of kynurenine to anthranilic acid, and of 3-hydroxy-

kynurenine to hydroxyanthranilic acid. A purified kynureninase has now been obtained from *Neurospora* (147), which also is pyridoxal phosphate-dependent, which converted kynurenine, 3-hydroxykynurenine, and N-formyl-L-kynurenine to anthranilic, 3-hydroxyanthranilic and formylanthranilic acids respectively. The kynureninase was strongly inhibited by many amines (148). The inhibition could be reversed by pyridoxal phosphate, possibly as a result of the formation of Schiff bases with the amines. However, both the substrate and the coenzyme were also inhibitory. The mechanism was not clear; the inhibition could be attributable to the substrate-pyridoxal phosphate compound. The latter acted as an inhibitor (148) in the kynureninase system.

Riboflavin was previously suggested to be involved in the conversion of kynurenine to 3-hydroxykynurenine (149). This does not appear related to the fact that the excretion of tryptophan metabolites was in the form of hydroxykynurenine in tuberculous patients (150) and in fever (151), but in the form of kynurenine in normal humans (151). No significant difference was found (152) in the metabolism of anthranilic acid by normal or riboflavin-deficient rats, whereas deficient rats after tryptophan administration excreted considerably more anthranilic acid and its derivatives than did the normal rats. These observations seem to indicate that riboflavin deficiency causes a derangement of the metabolism of tryptophan which brings about an excessive formation and excretion of anthranilic acid and its conjugates. A series of detailed studies were carried out (153) on the urinary metabolites after tryptophan and anthranilic administration in normal, riboflavin-deficient and riboflavin- and pyridoxine-deficient rats. Although riboflavin deficiency greatly disturbed the tryptophan metabolism with increased yields of xanthurenic acid, the formation of hydroxylated metabolites was not inhibited at all. From the available data, the authors suggested that kynurenine was



Structure XIV. Hydroxykynurenine phosphate.

oxidatively phosphorylated to hydroxykynurenine phosphate, XIV, and that the phosphorylated intermediate would normally be directed toward the formation of more hydroxyanthranilic acid and less xanthurenic acid. It was considered that riboflavin was involved in a phosphorylative, rather than an oxidative step in the disposition of kynurenine. This idea is in line with the findings (154) that tryptophan and kynurenine were converted to hydroxyan-

thranilic acid phosphate in rat liver preparations although hydroxykynurenine formed hydroxyanthranilic acid. Precise knowledge on the role of riboflavin in tryptophan metabolites will probably have to await the success of suitable *in vitro* experiments

Acetate or thioctic acid reversed propionate inhibition in *Streptococcus faecalis* in the presence and absence of riboflavin. In the presence of increasing amounts of lyxoflavin, extra riboflavin was necessary to restore the inhibition by thioctic acid (155, 156). It was concluded that L-lyxoflavin was competitively inhibitory to riboflavin in the organism. It is interesting to note that in the absence of the inhibitor (propionate), high dosages (up to 3 μ g per ml.) of lyxoflavin did not show any depression in growth. Thus, the reason why the competitive inhibition between these two flavins was demonstrable only in the presence of propionate remains to be discovered.

According to Snell *et al.* (157), different organisms showed different relationships between lyxoflavin and riboflavin. For example, L-lyxoflavin, D-galactoflavin, and isoriboflavin all increased the growth response of *Lactobacillus casei* to suboptimal amounts of riboflavin but had no effect in its absence. When riboflavin was present in excess, lyxoflavin did not increase the cell yield or growth rate. At high concentrations of lyxoflavin and galactoflavin, they inhibited the growth of the organism, as competitive antagonists of riboflavin. Both dichlororiboflavin and isoriboflavin were ineffective as inhibitors. L-Lyxoflavin promoted maximum growth of *Lactobacillus lactis* in the absence of riboflavin, and such growth continued indefinitely upon subculture. The cells from these cultures did not contain riboflavin, but lyxoflavin. It appeared, therefore, that in this organism, lyxoflavin could replace riboflavin in the latter's essential metabolic roles. In chicks, lyxoflavin stimulated growth when fed 2.5 to 10 times the amount of riboflavin in the diet. At high levels (over 40:1) lyxoflavin inhibited growth. The inhibition could be reversed by increasing the riboflavin content in the diet. A somewhat different result was obtained by Sherwood *et al.* (158), who found that although L-lyxoflavin was inactive in chicks, it stimulated growth of male turkey poults on a corn-soybean diet. Judging from the different responses to lyxoflavin shown in animals (111) and in microorganisms depending upon slight variances in media, the deviation of the results described is not surprising. The metabolic relationship between lyxoflavin and riboflavin is at the present time far from clear. However, L-lyxoflavin can hardly be considered as a vitamin in spite of the earlier favorable findings (111).

A review on metabolic functions of riboflavin has appeared (159).

VITAMIN B₆

The metabolically active form of the vitamin B₆ group is evidently the phosphoric ester of the 5-hydroxymethyl compound. If this hydroxy group is missing, the phosphoric ester can no longer be formed, although the intact hydroxymethyl group in position 4 could presumably still take part in the normal biochemical reactions. Consequently, the 5-desoxy compounds would

be expected to have no vitamin activity and might be inhibitors (160). For these reasons, the 5-desoxy analogues of pyridoxine, pyridoxal, and pyridoxamine were prepared (160) by a method illustrated in Figure 4.

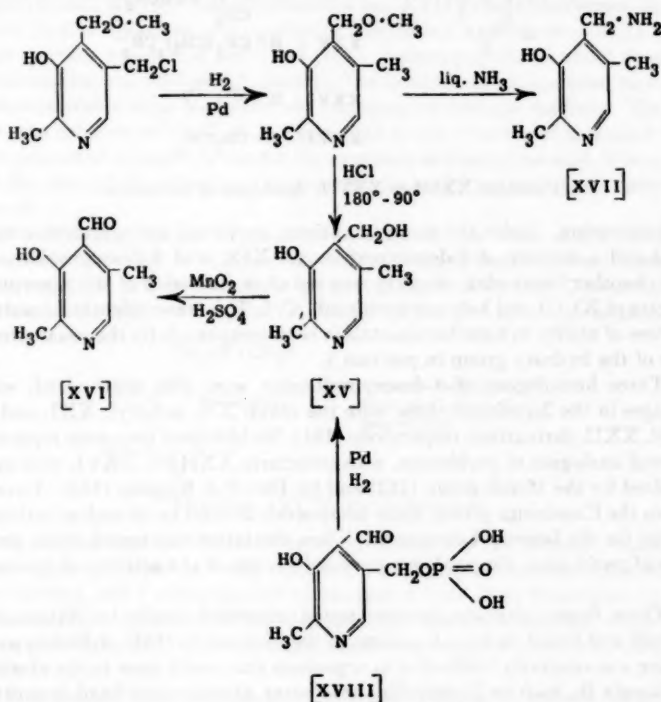
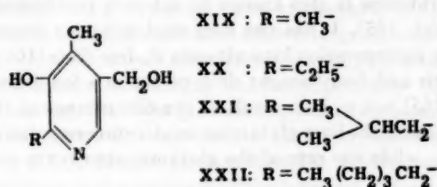
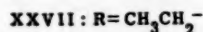
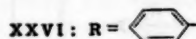
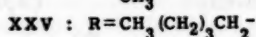
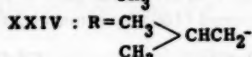
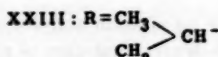
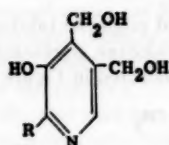


Fig. 4. Formation of 5-desoxy analogues of Vitamin B₆.

It is interesting to note that pyridoxal-5-phosphate, XVIII, could be directly hydrogenated to 5-desoxypyridoxine, XV. XV was resistant to further



Formulas XIX to XXII. 4-Desoxypyridoxine and its homologues.



Formulas XXIII to XXVII. Analogues of pyridoxine.

hydrogenation. Under the same conditions, pyridoxal and pyridoxine each produced a mixture of 4-desoxypyridoxine, XIX, and 5-desoxypyridoxine, XV. Another interesting property was the close similarity of the absorption spectra of XVIII and 5-desoxypyridoxal, XVI. This was evidently a result of the loss of ability to form hemiacetals in both compounds by the usual alteration of the hydroxy group in position 5.

Three homologues of 4-desoxypyridoxine were also synthesized, with changes in the 2-position: these were the ethyl, XX, isobutyl, XXI, and n-amyl, XXII, derivatives, respectively (161). No biological tests were reported. Several analogues of pyridoxine, with structures XXIII to XXVI, were synthesized by the Merck group (162) and by Davoll & Kipping (163). According to the Cambridge group, these compounds showed no biological activity, except for the isopropyl compound. When the latter was tested in the presence of pyridoxine, the analogue gave only 1/400 of the activity of pyridoxine.

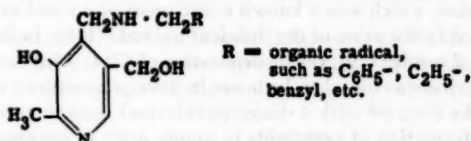
These desoxy compounds were tested microbiologically by Rabinowitz & Snell and found to be antagonists to the vitamin B_6 (164). 4-Desoxypyridoxine was relatively ineffective in organisms that could grow in the absence of vitamin B_6 , such as *Lactobacillus arabinosus*. On the other hand, it greatly impaired the growth of vitamin B_6 -dependent organisms, such as yeasts and molds (but not *L. casei*, *S. faecalis*, or *L. helveticus*). The comparative effectiveness of the various forms of the vitamin to counteract the inhibition was found to vary from one microorganism to another.

4-Desoxypyridoxine is also known to act as a pyridoxine antagonist in higher animals (cf. 165). It has also been used to hasten the onset of deficiency symptoms by incorporation into vitamin B_6 -free diets (166). However, the severe dermatitis and body weight drop of the rats fed 4-desoxypyridoxine were claimed (165) not to be a result of the disturbance of the transamination systems. Indeed, the liver glutamate-oxalacetate reaction remained practically the same, while the rate of the glutamate-pyruvate system increased markedly.

5-Desoxy analogues of vitamin B_6 were also antagonistic to vitamin B_6 in various microorganisms (167), as expected (160). The degree of activity was

different in different organisms. For example, the decreasing effectiveness as inhibitor in *Saccharomyces carlsbergensis* was approximately "ethyl" pyridoxine, XXVII > 4-desoxypyridoxine, XIX > 5-desoxypyridoxine, XV \geq 5-desoxypyridoxal, XVI > 5-desoxypyridoxamine, XVII. The effectiveness of reversal in this organism was pyridoxine > pyridoxal > pyridoxamine. In contrast to other pyridoxine inhibitors (164), 5-desoxypyridoxal and 5-desoxypyridoxamine also inhibited *S. faecalis*. The inhibition was, however, no longer demonstrable when D-alanine was incorporated into the medium. The result was interpreted (167) to indicate that in the absence of D-alanine the sole purpose of vitamin B₆ was for the synthesis of this amino acid. When the sole function of the vitamin was bypassed, the inhibitors no longer depressed growth.

The Merck group observed that several pyridoxylamines, XXVIII (168) were fairly active for rats. However, most of these compounds were found to have negligible activity for *S. carlsbergensis*, *S. faecalis*, and *L. casei* (169). Pyridoxylethylamine (R = ethyl in XXVIII) was the only one to show as



Structure XXVIII. Pyridoxylamines.

much as 2 per cent of the activity of pyridoxal. For *N. sitophila*, many of these compounds had low activity except ethyl, benzyl, methyl, ethanol, β -phenylethyl, and 3-phenylpropyl which showed from 20 to 90 per cent equivalent activity. It was recalled that pyridoxylamino acids also exhibited some activity in the mold, although inactive in yeast or lactic acid bacteria (170).

Functions of vitamin B₆ in metabolism are believed to include coenzyme activity for transaminases, dehydrases and desulhydrases, amino acid decarboxylases, racemases (159), thionase (171), and some other miscellaneous reactions. (Further details of the behavior of these enzymes should be consulted in the appropriate chapters on metabolism.) At one time transamination was thought to be limited to only two reactions, viz. aspartic plus α -ketoglutaric acid and alanine plus α -ketoglutaric acid. Now a large number of amino acids has been shown to participate in transamination (cf., for example, 172, 173, 174). Favorable evidences concerning the participation of B₆ in these reactions are still appearing. The liver glutamate-pyruvate system appeared to be more sensitive than glutamate-oxalacetate to dietary deficiency of vitamin B₆ in the rat (166, 174a); this also confirmed earlier Russian work. Glutamate-pyruvate transaminase and cysteine desulhydrase activities were significantly reduced in livers of the vitamin-deficient rat, while the glutamine-pyruvate and glutamine-phenylpyruvate reactions proceeded at approximately equal

rates (166). The impairments were corrected by the addition of pyridoxal phosphate to the preparations. The glutamine systems were shown to be unaffected because of the strong affinity of the coenzyme for the protein. However, a marked fall of the fasting level of plasma glutamine and a significant increase of blood urea were found in the deficient rat (175). Transamination of lysine was claimed to be unaffected by dietary deficiency of B_6 (176). This apparent independence might well be attributable to the affinity of the apoenzyme as in the case of glutamine.

Thionase (171), which can attack various thioethers, decreased rapidly in livers of rats on a vitamin B_6 -deficient diet (177). Addition of casein or sulfur-containing amino acids aggravated the effect. Incorporation of pyridoxal into the diet or addition of pyridoxal phosphate to the liver preparations restored the enzymic activity. This experiment supports the claim that this class of enzyme is pyridoxal-dependent (171). L-Cysteic acid was converted anaerobically to taurine by rat liver preparations. This conversion was diminished by the preparations from rats on a B_6 -deficient diet. This was in line with the fact that taurine, which was a known constituent of normal rat urine, could not be detected in the urine of the deficient animals (178). In line with a previous microbial result (179), the incorporation of C^{14} -labeled formate into the β -carbon of serine was found to be lower in liver preparations of pyridoxine-deficient chicks (treated with 4-desoxypyridoxine) than in normals (180).

The transformation of keto acids to amino acids in growing microorganisms has been studied extensively (181) and found definitely dependent upon vitamin B_6 . A clear-cut demonstration was also shown *in vivo* (182) in higher animals. Rats on a diet deficient in vitamin B_6 and an individual essential amino acid deficient diet did not respond to the addition of either the vitamin or the keto analogue of the amino acid. The addition of both resulted in a prompt stimulation in growth comparable to those obtained in rats on a complete diet. α -Hydroxy and keto-isovaleric and isocaproic, β -imidazole pyruvic and β -indole pyruvic acids each were able to replace the corresponding amino acid only when B_6 was present. From a comparison in regenerating liver tissue of partially hepatectomized, pair-fed control, and vitamin B_6 -deficient rats, the vitamin deficiency did not alter significantly the constituents, including glutamic dehydrogenase, either qualitatively or quantitatively. Vitamin B_6 was, however, found to be mobilized to the liver from extrahepatic tissue under conditions of rapid synthesis of liver protein in partially hepatectomized rats (183). No difference was found, likewise, between the normal and deficient rats in the absorption of protein, fat, and carbohydrate (184). Although nitrogen retention in the deficient rat was found to be less, the animal was able to maintain body protein (175, 184).

The mechanism of B_6 participation in enzymatic systems has received further attention. An attractive theory which was considered in earlier work (170) included a Schiff's base of structure XXIX as an intermediate, somewhat as shown in Figure 5. However, pyridoxyl glutamic, XXX, and pyridoxylidene glutamic acid, XXXI, were found completely devoid of activity in a

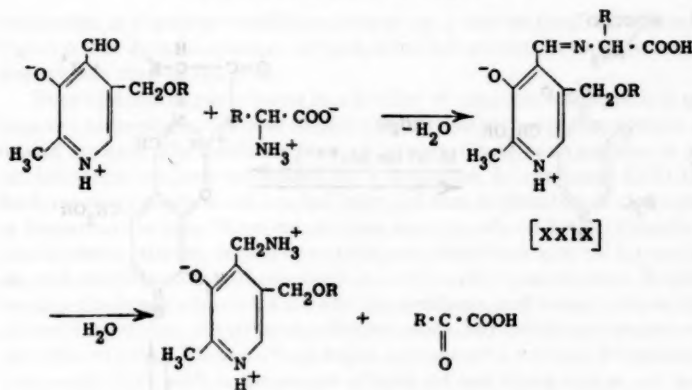
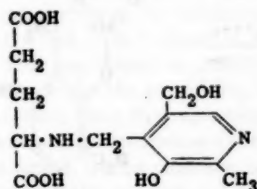
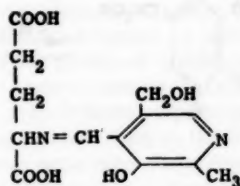


FIG. 5. Transamination via Schiff base formation.

highly purified preparation of glutamate-oxalacetate transaminase from pig heart (187). Compounds with structures resembling XXIX evidently cannot serve as intermediates in enzymatic transaminations unless the phosphory-



XXX. Pyridoxyl glutamic acid.



XXXI. Pyridoxylidene glutamic acid.

lated form shows a different behavior. Recently nonenzymatic pyridoxal- and metal (Cu^{++} , Fe^{+++} or Al^{+++}) catalyzed cleavages of hydroxyamino acids (159, 188, 189) were found to be demonstrable at pH 3 to 12 at room temperature. Isolation of chelates with Schiff bases containing pyridoxal, amino acid and metal ion were reported (190). These as well as other observations on nonenzymatic reactions led Snell to propose the reaction sequence shown in Figure 6 as the common feature of pyridoxal-catalyzed reactions (159). He stated:

... and resulting in an activation of the amino acid residue so that it may undergo any of a variety of reactions in groupings immediately surrounding the α -carbon atom. ... The vacant coordination positions about the metal ion might well serve to tie the coenzyme (and substrate) to the apoenzyme; in non-enzymatic reactions, these coordination positions are undoubtedly occupied by water or additional molecules of the reactants.

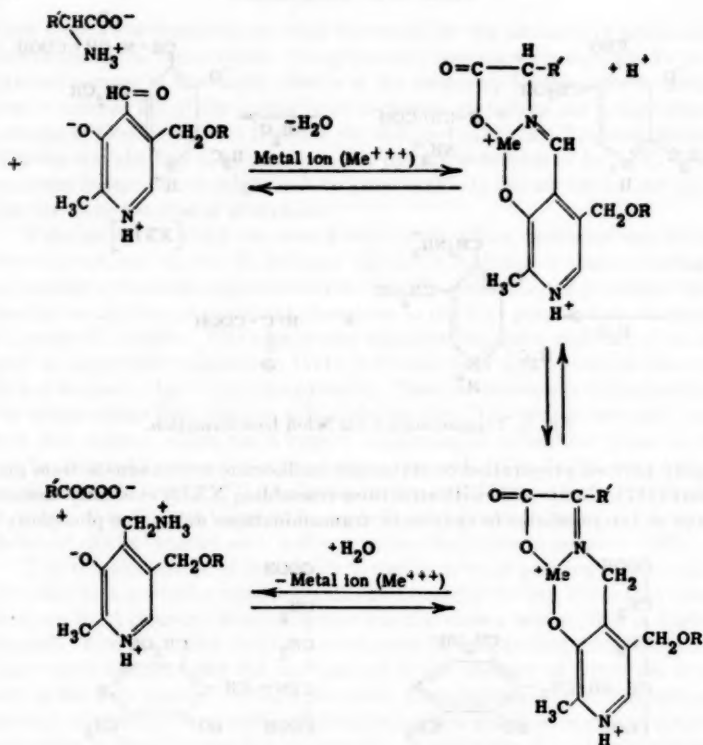


FIG. 6. Transamination via chelate formation.

The test of this hypothesis in enzymic reactions would be of interest. However, it should be pointed out that the phosphorylated vitamin is always required in enzymic reactions while the nonphosphorylated form can catalyze the nonenzymatic reactions. If, as seems logical, this fact indicates an attachment to protein through the phosphate bridge, then the 5-desoxy analogues should not be able to compete with the vitamin for the protein, i. e., they should remain inert, unless another bond between the coenzyme and protein is proposed. It will be seen that the mechanism in Figure 6 calls for the presence of a hydrogen atom on the α -carbon of the amino acid. α -Methylglutamic acid, in which no α -hydrogen exists is a potent inhibitor of glutamic decarboxylase and can react with pyridoxal phosphate nonenzymatically (191). Presumably, the breakdown of normal enzymatic activity is caused by the need for a mobile hydrogen; yet it has been reported that the α -hydrogen in glutamic acid is probably not labilized during decarboxylation (192), whereas

labilization of this atom would appear to occur in the reactions represented in Figure 6. For decarboxylation, at least, some refinements in this new attractive scheme may be required.

Since vitamin B₆ participates in a number of catalytic reactions, it is perhaps not surprising to discover further connections with some apparently unrelated systems. The similarity in syndromes attributable to pyridoxine and fat deficiency has been recognized for a long time. It was found (185) that linoleate was a precursor of arachidonate and that linolenate was a precursor of hexaenoate in rats. These conversions were stimulated by B₆. Vitamin B₆ plus linoleate relieved dermal symptoms and stimulated growth, fat synthesis, and arachidonate synthesis much more than either supplement. Pyridoxine plus linolenate stimulated growth, fat synthesis, and hexaenoate synthesis much more than did either supplement alone, but dermal symptoms were not relieved (185). These findings might account for a marked diminution in total crude fatty acids in the carcass of both old and young rats on a vitamin B₆ deficient diet (184, 186). When sodium salts of fatty acids were administered to rats together with tryptophan, the excretion of xanthurenic acid in the urine was greatly enhanced. Such increment could be checked by injection of pyridoxine (187a).

An interesting report has appeared concerning the relationship between pyridoxine and the gonadotrophic content of the anterior pituitary (188a). Rats on a vitamin B₆ deficient, 4-desoxypyridoxine containing diet had a great increase (5 to 8 times) in the gonadotrophic content of anterior pituitary. This was almost entirely a result of the change of follicle-stimulating hormone, while the luteinizing hormone content remained practically the same (188a). On a B₆-supplemented diet, growth hormone promoted weight gain and nitrogen retention while it did not promote these on the vitamin deficient ration. On the contrary, the hormone aggravated the symptoms of B₆ deficiency, such as increased severity of acrodynia, a further elevation of blood urea, and a further lowering of liver B₆ content (189a). This observation should belong to the same category as the one usually used in nutritional experiments which uses thyroid or iodoproteins to increase metabolic rate. The increase in metabolism can accelerate the depletion of a limiting dietary factor to be examined so that the hypophysectomized vitamin B₆-deficient rat does not show external signs or biochemical evidence of deficiency (189a).

The formation of indole, as well as the decarboxylating activity for arginine, ornithine, and glutamic acid in *E. coli* was decreased by treating resting cells with isonicotinic acid hydrazide. The inhibition could be reversed by addition of pyridoxal to the system (190a).

The aldehyde group of pyridoxal could be oxidized in the presence of liver aldehyde oxidase to pyridoxic acid. This reaction was observed in rat and rabbit liver preparations (191a). The significance of the reaction remains to be investigated.

A differential method for determination of pyridoxine, pyridoxal, and pyridoxamine was reported. A preliminary separation was based on the basicity

of pyridoxamine, and lability of pyridoxal (192a). A review of metabolic functions of vitamin B₆ appeared (159).

NIACIN

It is generally agreed that in many organisms tryptophan is a precursor of nicotinic acid, although the yield from the conversion is very low. Besides serving as a precursor of the vitamin, tryptophan is also one of the building blocks of proteins. The biosynthesis of protein is, however, a complex process which is dependent upon many factors, including the simultaneous availability of amino acids. It is logical to believe, and it has been demonstrated (cf. 193) that this synthesis is an all-or-none operation. Consequently, an intriguing interrelationship exists between these two biosynthetic processes. A metabolic priority, if any, can be revealed by a suitable plan of experiment.

Increase of essential amino acids in diets containing marginal levels of tryptophan should be able to induce niacin deficiency by funneling the tryptophan toward protein synthesis. Henderson *et al.* found this to be true (194) by using an ingenious adjustment of the dietary amino acids. Growth of rats was severely suppressed by increasing threonine from 0.33 to 0.38 per cent of a diet which contained 10 per cent hydrolyzed casein and 0.1 per cent tryptophan. Levels of lysine above 0.5 per cent and valine above 0.7 per cent caused a growth suppression which could be corrected by niacin. Similar results were observed in a 10 per cent zein diet (194, 195). The observations also revealed that under these conditions tryptophan was preferentially used in protein synthesis at the expense of niacin formation. Thus in the establishment of the nutritional requirement, the amino acid levels should be considered (see also 196).

Many apparently conflicting results were obtained in studying niacin metabolism in different phyla. As pointed out (197), mammals may be divided into two groups in respect to niacin metabolism: one group, including man, dog, and cat, can aminate nicotinic acid, whereas the other, including the guinea pig and rabbit, deaminates nicotinamide to nicotinic acid. However, N'-methyl nicotinamide (NMN²) is very unstable in alkali. The alkalinity of rabbit urine may destroy the compound in the urinary tubules before it is excreted. It was found that the administration of mandelic acid to increase the urinary acidity enhanced the excretion of NMN in rabbit (197). Besides NMN, nicotinic acid, nicotinuric acid, and N'-methyl-2-pyridone-5-carboxylamide were also detected. The last formed the principal end-product of nicotinic acid metabolism. By an elaborate technique of chromatography, Reddi & Kodicek, however, were not able to detect the pyridone in man and rat after administration of nicotinic acid, nicotinamide, or tryptophan (198). After a dose of 100 mg. nicotinic acid to man, the main end-products found in urine were nicotinuric acid > NMN > nicotinamide. Nicotinic acid did not appear in the urine until vasodilatory symptoms occurred. The pattern was the same in the rat, except that nicotinic acid was a normal constituent. After nicotinamide administration, nicotinuric acid was not regularly found in either species, otherwise the excretion was the same as after nicotinic acid (198). By

using bioautography and radioautography on *Torula cremoris* cultures, Johnson & Lin (199, 200) found essentially the same results following large doses of carboxyl- C^{14} labeled vitamin in rats. Nicotinic acid and NMN-like compounds accounted for 60 per cent of the C^{14} excreted after administration of nicotinic acid and nicotinamide respectively. At least seven and nine other products, respectively, were found in the urine to have radioactivity after nicotinic and nicotinamide addition. Of these unidentified compounds, the chief one followed the same chromatographic behavior as N'-methyl-2-pyridone-5-carboxylamide, which the Cambridge workers were unable to find.

In contrast to the rabbit and other mammals, the urinary excretion of NMN could not be detected following the subcutaneous injection of nicotinamide into either a herbivorous (*Bombyx mori*) or a carnivorous insect (*Lucilia caesar* L.) (201). Niacin metabolism has thus been shown not only to exhibit species differences but also to vary according to hereditary factors (202). Two pure strains (albino and black hooded) of rats showed great differences in their daily elimination of nicotinamide methochloride and their response (i. e., the increase of this excretion) to extra dietary administration of nicotinamide. Both factors were remarkably stable. When they were crossbred, the output of the methochloride and the response to extra nicotinic acid were inherited multifactorially. The two sets of genes controlling the two phenomena had some, but not all, members in common. No evidence was obtained for a direct effect of sex, or that sex-linked genes, played an important part in the heredity of output and response (202). These studies on the end products contribute significantly in the advance of the knowledge of intermediary metabolism of the vitamin. The observations after large dosages, as pointed out with thiamine, should, however, be carefully regarded before application is made to normal metabolism. Other types of experimentation such as *in vitro* studies of purified systems, may provide the information that is needed, based on the urinary inventories.

Acetylpyridine has been recognized for some time as an inhibitor of niacin in mice. The same compound has recently been suggested as a precursor of the vitamin. 3-Acetylpyridine was converted in the adult dog to nicotinamide, NMN, and nicotinic acid, in yields of 20 to 30 per cent (203). At high levels (0.1 gm. per kilo of ration) however, acetyl pyridine was found very toxic to the animal. Further studies (204) showed that the conversion took place rapidly in blood cells, liver, and kidney slices, but not in muscle or blood plasma. The action of acetyl pyridine as a precursor was confirmed (205) by the finding that a daily dose of 60 mg. could prevent the symptoms of blacktongue in dogs on a niacin deficient diet.

Nicotinic acid or its ethyl ester was not used in the biosynthesis of nicotine by incubating the compounds with tobacco root, even over a period of five weeks (206). A summary of the metabolic functions of nicotinic acid appeared (159).

ASCORBIC ACID

Evidence on the mechanism of ascorbic acid biosynthesis has been provided in the recent work by C. G. King, Isherwood, and their colleagues. The

Columbia school has employed the chloretone-treated rat. Chloretone increased ascorbic acid synthesis, and thus facilitated the characterization. Ascorbic acid isolated from the urine after administration of uniformly labeled C^{14} -glucose was uniformly radioactive (207). Glucose-1- C^{14} gave ascorbic acid containing C^{14} chiefly in position 6 (208), whereas glucose-6- C^{14} led principally to ascorbic acid-1- C^{14} (209). The suggestion of direct conversion of D-glucose to L-ascorbic acid induced the authors to study glucuronic acid in the biosynthesis. By using uniformly labeled glucuronolactone, a uniformly labeled ascorbic acid was obtained. The yield in terms of radioactivity from glucuronolactone was many times greater than that from glucose (210). The formation of glucuronolactone from glucose was previously demonstrated in the rabbit *in vivo* (211), and in guinea pig liver *in vitro* (212). The synthesis thus might be summarized as: D-glucose \rightarrow D-glucuronolactone \rightarrow L-ascorbic acid. The second step is actually an intramolecular oxidation-reduction and was thought possibly not to need more than one enzyme (210).

Although this scheme was developed in the chloretone treated rat, chloretone *per se* was not a precursor of the vitamin. Moreover, Isherwood *et al.* employed the normal rat with similar results. The Cambridge workers (213) discovered that not only the γ -lactones of D-glucuronic acid, XXXIII, and L-gulonic acid, XXXIV, when fed to cress seedlings or injected into rats, were transformed into L-ascorbic acid, XXXV, but also D-galacturonic acid, XXXVII, methyl ester and L-galactonic acid γ -lactone, XXXVIII. No other sugar acid γ -lactone tested yielded L-ascorbic acid. L-Idono- γ -lactone, XXXIX, and L-talono- γ -lactone, which differ from L-gulono- and L-galactono- γ -lactones only in the configuration of carbon 5 (or 2'), were inactive. This fact led these investigators (213) to formulate two analogous sequences in the biosynthesis of ascorbic acid as shown in Figure 7.

A fundamental feature is the change from the D- to L- series at the second step with inversion of the whole molecule. The enzyme or enzymes catalyzing the transformation of the aldonic acid into L-ascorbic acid is specific for a D-configuration of the hydroxyl group on C-4 and an L-configuration of the hydroxyl groups on carbons 2' and 5' referred to ascorbic acid.

The configuration of carbon-3' is also specific; thus D-araboascorbic acid, XLIII was produced in the rat from D-mannono-lactone, XLI, and in cress seedlings from D-altrono- γ -lactone only (Fig. 8). However, no similar difference was found in L-gulonic and L-galactonic acid- γ -lactone. It appears that organisms such as the guinea pig, which require the exogenous vitamin, are lacking the enzyme or enzymes to perform the second step.

The report by Nath *et al.* (214) raised a question whether the Isherwood schemes were the only pathways leading to ascorbic acid, although the Cambridge group did not find any evidence of involvement of L-glyceraldehyde, D-gluconic acid, sorbose, or sorbitol. Acetoacetate could decrease the vitamin in the animal (215) but induced a slight increase of ascorbic acid when administered simultaneously with glucose. This effect was also noticed in germinating mung beans (*Phaseolus mungo*). However, a much more striking increase was demonstrated in both organisms after administration of a conden-

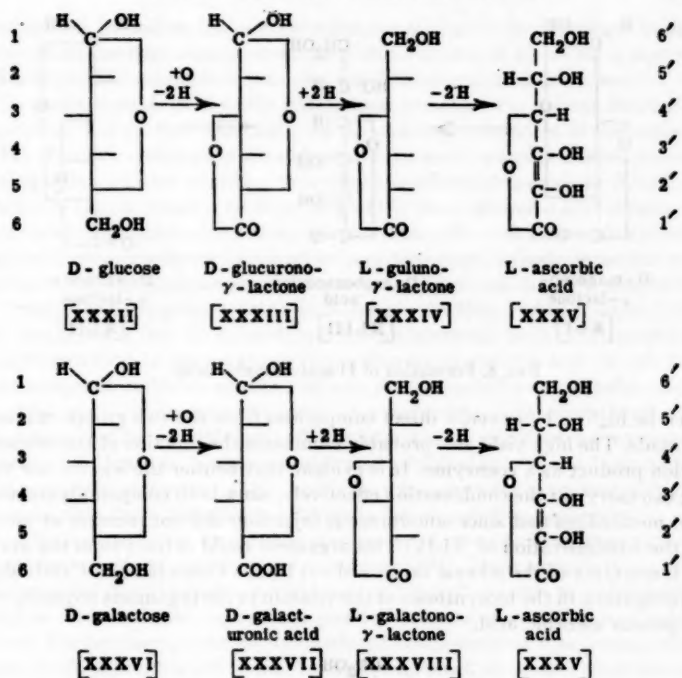
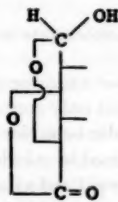
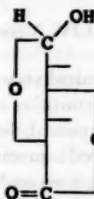


FIG. 7. Biosynthesis of L-ascorbic acid

sation product of these compounds. This product was believed to have the structure shown in XLIV. One hundred mung bean seeds gave 2.79, 4.12, and about 2.1 mg. of ascorbic acid in a medium containing supplements of 5 mg. of XLIV, 25 mg. of XLIV, and none, respectively. The conversion (about 10 per cent) of the condensation product to ascorbic acid was rather high relative to the experiments by Horowitz *et al.* (210). Since the Indian authors did not report the method and purity of the preparation, the actual yield might



Structure XXXIX. L-idono-γ-lactone



Structure XL. L-talono-γ-lactone

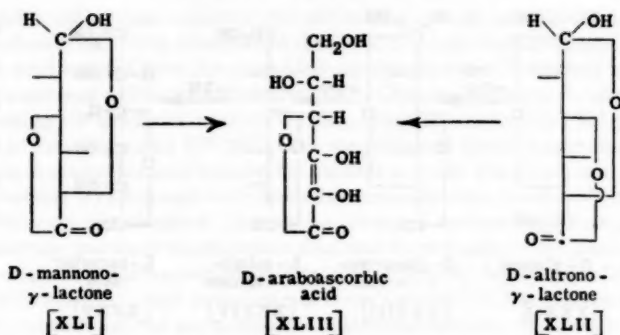
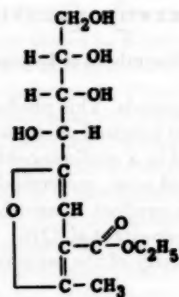


FIG. 8. Formation of D-araboascorbic acid.

even be higher. However, a direct comparison from the two groups can not be made. The high yield also probably eliminates the function of the condensation product as a coenzyme. It is evident that neither the legume nor the rat can carry out the condensation effectively, since both compounds are normal metabolites and since simultaneous injections did not increase as much as the administration of XLIV. This argument could detract from the overall importance of the scheme as worked out by the Columbia and Cambridge investigators, in the biosynthesis of the vitamin in the organisms requiring no exogenous ascorbic acid.



Structure XLIV. A condensation product of acetoacetate and glucose.

From the chemical viewpoint, ascorbic acid has a unique structure among vitamins, since it contains a dienol group which not only contributes reducing action to the compound, but also confers an acidic behavior upon the molecule. In nonbuffered aqueous solution it was oxidized by *o*-iodosobenzoic acid, the rate following a second order reaction. The oxidized vitamin underwent transformation to an equivalent molar quantity of a product which was read-

ily oxidized by iodine but not by iodosobenzoic acid. In phosphate buffer, more extensive degradation occurred with formation of up to three equivalents of products capable of reducing *o*-iodosobenzoic acid (216).

D-Araboascorbic acid which differs from ascorbic acid only at the configuration of C-5 showed practically all the chemical properties of the vitamin (217). The two compounds showed almost the same cyanide- or phenol-combining ability. Either could prevent the toxic effects of cyanide in *E. coli*, of phenol in rats, or tetanus toxin in mice (217). Both showed a stimulatory effect for the formation of citrovorum factor from folic acid. Conversion of folic acid to citrovorum factor involved at least two steps, namely reduction and formylation. The nonspecificity of the reducing agent was shown in mice (217), in chick liver homogenates (218), in *S. faecalis* A (219), and in *L. casei* (220). In fact, tetrahydro- (221) or dihydropteroylglutamic acid (222) might be substituted for citrovorum factor in the absence of ascorbic acid or other reducing agents. D-Araboascorbic acid was demonstrated to be equally potent in most of the above investigations as a reductant. The pantothenic acid sparing action by ascorbic acid (223) could also be exhibited by D-araboascorbic acid. However, D-araboascorbic acid exhibited only about 5 per cent of the antiscorbutic potency (217), and it therefore does not seem reasonable to propose that the antiscorbutic activity is due to reducing properties.

The first oxidative step of the conversion of tyrosine to acetoacetic acid had been considered ascorbic acid dependent (224). More recently it has been shown (225) that the reaction could use, with equal activity, D-araboascorbic acid or hydroquinone, in acetone powder extracts of rat, dog, and rabbit livers. Furthermore, structurally unrelated compounds such as homogentisic acid, PAB, *p*-phenylenediamine, 2,6-dichlorophenolindophenol could also increase the tyrosine oxidation. The positive correlation of blood vitamin A levels with plasma ascorbic contents (226) might well be attributable to the antioxidant property of the vitamin. An observation (227) was reported that ascorbic acid inhibited the proteolysis by crude pepsin preparation but did not by crystalline pepsin. The reason might be attributable to inactivation of impurities in the crude preparations that were dependent upon the redox behavior of ascorbic acid.

The literature concerning the replacement by ascorbic acid of the vitamin B₁₂ requirement in lactic acid bacteria has been reviewed recently (228). It was suggested that ascorbic acid functioned by reactivating inactive oxidized fragments of vitamin B₁₂ (229). The observations (102) of the growth of *L. fermenti* in a thiamine-free but ascorbic-containing medium might be an analogy of the B₁₂ case. In these experiments on animals as well as microorganisms, the only requirement may well be simply a compound with a proper redox potential.

Some functions, however, exhibited by ascorbic acid are not easily explainable by its reducing action, but on the other hand are also obscure from a purely enzymological view. Adequate ascorbic acid intake was shown to be required not only for normal healing in the early post-wound period in the

guinea pig, but also for the maintenance of the scar tissue in later stages (230). This might be a result of an increased ascorbic requirement during the formation of collagen-containing tissue, as observed in the same animals (231). The vitamin deficiency also caused the impairment of VEM (vasoexcitator material) (232) and decrease of liver protein content (233). The mechanism of stress is not very clear and the role of ascorbic acid in stress (234, 235, 236) is more obscure.

In 1951, Long and associates (237) found that more cholesterol was deposited in the adrenals of ascorbic acid deficient guinea pigs than in those of the normal controls. This fact was also reported by King *et al.* (238). Ascorbic acid-1- C^{14} was not appreciably incorporated into cholesterol. The vitamin exerted a marked effect upon the conversion of C-1 labeled acetate to cholesterol and other steroids in guinea pig *in vivo*. Compared with *ad libitum* and pair fed controls, a progressive increment in their fixation of acetate in cholesterol with the onset of scurvy was observed. The severely scorbutic animals incorporated six times as much C^{14} from acetate into cholesterol isolated from adrenals (238, 239). This fact may be explained by assuming that either the metabolism of acetate is deranged or the further utilization of sterols is depressed by the lack of ascorbic acid. It has been suggested by several groups that the biosynthesis of cortical hormones is inhibited by ascorbic acid deficiency (240). If this hypothesis is correct, cholesterol should obviously accumulate. Many previous negative results might be a result of difficulties in methodology. Recent findings (241) showed that ascorbic acid depressed the breakdown of cortical hormones to 17-ketosteroids determined as dehydroisoandrosterone. Simultaneous ascorbic acid treatment of adrenalectomized rats receiving cortisone acetate resulted in a decrease of urinary 17-ketosteroids and an increase of urinary corticosteroids (241). The same authors also found (242) in *in vitro* experiments that liver tissue of ascorbic acid-treated rats metabolized the 17-OH, as well as the 20,21-ketosteroid, and the conjugated unsaturated systems of the corticosteroid structure at a slower rate than does liver tissue of control rats. The influence of ascorbic acid in sterol metabolism is further complicated by the effect of other compounds, such as insulin (243). Ascorbic acid enhanced the insulin tolerance of cortisone-treated, adrenal-demedullated rats. The vitamin *per se* did not affect insulin tolerance. The urinary glucose and nitrogen were increased after cortisone injection. These increases were further enhanced when ascorbic acid was used along with cortisone. This observation confirmed the view that ascorbic acid can enhance gluconeogenic action of cortical hormones. As a matter of fact the lack of the vitamin induced hyperglycemia in guinea pigs by repeated injection of acetoacetate (215). The injections also depleted glycogen storage in the deficient animal. No steroid determinations were made in this study. Possibly the steroid metabolism is also greatly disturbed, so that a gross abnormality was observed.

Certain oxidases, such as potato polyphenol oxidase (244) and xanthine oxidase (245), were inactivated by ascorbic acid. The incubation of purified

xanthine oxidase with trace amounts of ascorbic acid resulted in severe inhibition in the enzymic activity. However, blood levels and rate of excretion of uric acid and allantoin were independent of the ascorbic acid intake in guinea pigs. Contrary to expectations, when polyphenoloxidase was treated with ascorbic acid under anaerobic conditions, and the ascorbic acid was then removed by dialysis, the enzymic activity was greatly reduced. The treated enzyme could not be reactivated by cupric ions. No explanation was given. It would be of interest to determine whether the inactivation in both systems may be a result of the antioxidant action of the vitamin. On the other hand, an ascorbic oxidase of fungus spores was found to be completely specific (246). It was irreversibly inhibited by D-araboascorbic acid and was quite different from other described ascorbic oxidases being inert toward "copper poisons" and inhibitors for iron proteins (246, 247).

In spite of the many examples given in the foregoing pages which point to a relatively nonspecific reducing function for ascorbic acid, it seems compelling to regard this vitamin as specifically essential for other purposes. Thus, the scorbutic condition in the animal can not be non-specifically relieved by reducing agents. Such a specific role, however, should be referable to a participation in one or more enzyme systems, in a manner that can be interpreted biochemically. The present uncertainty regarding ascorbic acid function must continue to present a challenge to enzyme chemists to determine the proper place in the metabolic scheme for this important vitamin.

Improved methods for the determination were reported (248, 249), and reviews on the interaction of ascorbic acid with bacteria (228), its role in plants (250), and its physiological role in general (240) have appeared during the past year.

MISCELLANEOUS

The beneficial effect of antibiotics on animal growth was considered (251) to be attributable to an alteration of the intestinal flora; growth rates of both PA- and riboflavin-deficient rats were improved. This study confirms the general experience reported in a review (252), in which faster growth resulted when antibiotics were fed to calves, swine, turkey poults, and chicks, as well as to school children on all-vegetable diets. Sparing action of the growth promutant upon the dietary vitamin requirements was usually observed.

A purified diet was described for young guinea pigs (253) which was used for the production of specific vitamin deficiencies. It was composed of sucrose, corn starch, cerelose, cellophane, vitamin-free casein, corn oil, minerals, and vitamins. The complete diet was stated to support growth and physiological development as satisfactorily as the same diet supplemented with greens, or as a supplemented commercial rabbit chow. Omission of any of seven B vitamins (but not B₁₂, biotin, or inositol) produced clear-cut deficiencies of each.

Destruction of certain B vitamins within purified rations was traced to the salts being used (254): the Phillips-Hart mixture (255) employed was more deleterious than a U.S.P. XI formula. The effect was greatest on thia-

mine [see also Thiamine section (107)]. This was thought to be attributable to the alkalinity of the mixture rather than to the effect of specific ions.

New growth factors.—Research on new growth factors continues at a rapid pace. A recent tabulation (114) contained nearly 50 reports of still unidentified principles for animal or microbial growth or enzymatic action; since then, at least another 20 papers have appeared. Most of these factors are probably duplications, and others may likely be artifacts or represent the combined effects of already known factors. Nevertheless, some of these, such as the xanthine oxidase factor [inorganic molybdate (136, 137, 256, 257, 258); see Riboflavin section], coprogen (259), and ferrichrome (260) have been carried to the point where they are known to be specific entities in pure form. Ferrichrome, a growth factor for *Pilobolus kleinii* and *Arthrobacter terregens*, has been isolated from the smut fungus *Ustilago sphaerogena*. The pure crystalline compound is 500 times more potent than hemin, which was previously found to be a growth requirement for the organism (261, 262). Its provisional formula was assigned as $C_{27-29}H_{42-46}O_{12}N_9Fe$, with a molecular weight of 700 to 800 calculated from diffusion data. Its structure is not known, but a hydrolyzate of the compound was found to contain glycine and a dibasic amino acid (263). A specific spectrophotometric method of assay has been described, which permits the assay of 3 μ g. or more of the substance per ml., with a reproducibility of ± 4 per cent (264). It is interesting to note that addition of zinc to the smut fungus culture immediately reduces the ferrichrome concentration of the cells, while the metal is essential for the accumulation of cytochrome-c, as well as for rapid growth, in the same organism (265). The redox potential of oxidized-reduced ferrichrome was found to be considerably lower than that of cytochrome-c. The reduced form can be stabilized by cyanide to prevent air oxidation (261). Its role, if any, in electron transport, and its relationship with the cytochrome system and with coprogen (259) deserve additional study.

Another vitamin, B_{12} , has received further clarification (266) after a lapse of several years (267). It was originally concentrated from distillers' dried solubles. Orotic acid has now been isolated from that source and found to be active in the B_{12} (rat growth) assay. Based on this propinquity, together with a similarity in absorption spectra, the authors suggest (266) that B_{12} and orotic acid may be related, although not identical. The earlier work (267) reported growth responses from B_{12} with as little as 6 μ g. per day, whereas 1 mg. of orotic acid was required to produce a growth response (266).

Most difficult to evaluate are the factors which have been characterized only by a single growth or other physiological response. Into this category fall a score or more of "factors" that have been reported in recent years dealing with growth of chicks and poults. Opportunities seem excellent for duplication not only among each other but also with new forms of vitamin B_{12} , pyrimidines (whey factor), and with unidentified microbial growth factors. In addition, strain and environmental differences may operate to affect growth. In spite of these restrictions, it appears that two, and possibly three,

distinct factors for poultry nutrition may now be under study. These are: (a) a factor or factors in liver or fish solubles, active on chicks and poults, and in improving hatchability (268 to 277). This has been fractionated (278) from fish solubles and shown to be soluble in water and in phenol-water or methanol-water mixtures, but not in ether. It is reportedly not identical with lyxoflavin or dried whey. Concentrates were active at 0.05 per cent of the diet. Another fractionation study, however, states that the factor is insoluble in 72 per cent phenol (279), not adsorbed on Norite at acid pH and heat stable (280). (b) A factor from whey (270, 272, 281 to 284), which may also be present in such materials as soybean (285) or yeast (286). Possible relationships to orotic acid or B₁₂ suggest themselves (cf. also 287). A third possibility is (c) a factor in grass juice (288, 289). Whether the growth promoting effect of alfalfa meal (290) is related to the grass juice factor appears unknown. The enlarged hock disorder in turkeys (286) has been shown to be preventable by niacin and vitamin E (291).

Progress toward the identification of these factors has been relatively slow. This has no doubt been attributable to many causes, including the long period required for assay, the slight growth effects observable (often as low as 10 per cent over the growth on the basal diet), and the effect of infection, antibiotics, and even the physical environment on growth responses. Several workers have recorded better growth in clean cages or batteries than in quarters that have seen prolonged use. In addition, only a few of the studies cited have employed purified basal diets or have extensively concentrated the supplements being fed. Until this is done, uncertainty will persist regarding both the number and the nature of these stimulatory agents.

Other factors for higher animals include: an antipancreatic fibrosis factor for ducks (292) and for rats (293, 294). "Stress factors" have been reported for rats, which were required during feeding of thyroid extract or iodinated proteins; these were thought to respond to lyxoflavin (295), but other reports (296 to 299) have indicated a requirement for an unknown liver principle to overcome the thyrotoxic effect.

Some factors for bacteria and other lower forms include: N-D-glucosylglycine and related glycosylglycines, for lactic acid bacteria (300); stimulatory factors from marine algae extracts for both *S. faecalis* and *Leuconostoc citrovorum* (301); growth factors for *Lactobacillus bifidus*, including strepogenin fractions (302), poultry fecal extracts (303), papaic digest of soybean (304), as well as blood group mucoids (305, 306); histidine (307) and serine (308) peptides for *Lactobacillus delbrückii*; *Staphylococcus albus* growth factors which were previously thought (309, 310) to be special peptide structures, especially those of cystine, but which were later reported (311) to be active even after removal of all amino acids; a growth factor for *Fusobacteria* (312); alkali stable factors for lactobacilli, which may be related to B₁₂, but are thought not to be identical (313, 314); a growth factor for pleuropneumonia-like organisms, which is reported to be a low molecular weight basic protein (315); growth factors for Clostridia (316) which may be strepogenin-like; a

growth factor for *Trypanosoma cruzi*, which is believed to be a complex hemoglobin derivative (317); and a growth factor for Pythiogeton (318). Mycobactin (empirical formula: $C_{47}H_{73}O_{10}N_8$), which is essential for the growth of *Mycobacterium johnei*, has been isolated into a crystalline aluminum complex from a nonpathogenic *Mycobacterium* (319). Unknown materials, probably nucleotides, appear active as a coenzyme for alcoholic fermentation (320) or for citrate oxidation (321). A bacterial deaminase cofactor has been described which is produced by heating carbohydrates with H_2SO_4 (322). This decomposition product was thought to be functionally related to biotin and adenylic acid.

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NUTRITION^{1,2}

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An attempt will be made in this review to emphasize those areas of investigation in which noteworthy progress has been made. One of the outstanding difficulties encountered in assessing progress in nutrition during 1953 was the lack of standardization of diets used by laboratories when similar problems were under study. The gathering volume of data indicates the marked effect of small changes in the diet when the nutrient or the metabolic balance is critical. This is perhaps one of the most significant aspects of nutrition research with experimental animals during the past year. It is heartening that Turk (1), a leading soils authority, should "spike" the half-truths of the food faddist by stating, "The theory that the over-all nutritive value of foods for man is favorably influenced by a fertile soil has not been proved," which means that the qualitative character of foods is little affected by fertilizers applied to the soil.

CARBOHYDRATES

Ratio techniques for the estimation dry of matter consumption of grazing animals have been studied by Kane *et al.* (2). The method requires two factors: A measurable constituent present in the forage, such as lignin, plant pigment, or indigestible protein; and an indigestible substance such as chromium sesquioxide, barium sulfate, radioactive isotopes, or dyes. The following formulae were developed:

$$(A) \text{ Dry matter digestibility} = 100 - 100 \frac{\text{per cent indicator in the feed}}{\text{per cent indicator in the feces}}$$

$$(B) \text{ Dry matter consumption} = \frac{\text{Total amount of "external" indicator fed}}{\text{Amount of "external" indicator in feces}} \\ \text{multiplied by} \quad \frac{\text{Amount of dry matter in feces sample}}{\text{Per cent dry matter indigestibility}}$$

These authors have shown that the dry matter intake calculated by formula B, based upon chromium oxide and lignin, agreed closely with weighed amounts of dry matter fed to experimental animals and were equally valid with standard total collection procedures. This held for other internal factors as well as laboratory indicators. The results obtained were within plus or

¹ The survey of the literature pertaining to this review was completed in November, 1953.

² The following abbreviation is used: TDN for total digestible nutrients.

minus 2 per cent, by this method of measuring both dry matter consumption and digestibility of forage consumed by grazing animals. These authors point out that there is "no loss of dry matter on drying fecal residues at 80°C." Concurrently, Hardison *et al.* (3) verified the procedure of dye dilution in measurement of dry matter intake of grazing cattle.

Interest continues concerning the role of phosphorylation of sugars in their relationship to nutrition and absorption. Stable methyl ethers of glucose, i.e., 2,3,5-, and 6-methyl derivatives and their influence upon the absorption from the intestine of the rat was studied by Csaky (4). 3-Methyl glucose was selectively absorbed at the same rate as glucose, whereas the others were not. It was shown that the amount of free reducing sugar decreased in the case of both glucose and 3-methyl glucose incubated with rat intestinal mucosa and ATP (adenosinetriphosphate). No decrease took place if ATP was omitted. The author concludes that this is presumptive evidence that the disappearance of free-reducing sugars is attributable to phosphorylation by hexokinase. This position is supported by the work of Long (5). His data show that a high carbohydrate (fat-free) diet led to a significant increase of hexokinase activity of the intestinal mucosa of the rat and conversely, a carbohydrate-free (high fat) diet led to a reduction of high hexokinase activity of the intestinal mucosa. Differences were detected as early as one week and up to eight months.

The importance of citric acid in the diet and metabolism of the animal organism has been amply reviewed by Thunberg (6). He estimates that citric acid contributes 2.47 calories per gram and that it is completely consumed in the animal organism. He points out that bone, as well as semen samples contain considerable amounts of citric acid.

Lundt & Sutcliffe (7) demonstrated that resorcinol-4,6-disulfonic acid could be used as a new colorimetric reagent for the study of hexoses and their polysaccharides. Ten to 250 μ g. in 5 ml. could be determined within plus or minus 1 per cent.

Smith *et al.* (8) studied the glucose, fructose, and "invert sugar" tolerance in diabetic patients, and those with parenchymal hepatic disease. They found the rate of disappearance of fructose from the blood to be a first order reaction. The metabolism of fructose was impaired much less than that of glucose under the same conditions. Blood phosphorus levels fell more rapidly when fructose was used, with a concurrent rise in carbohydrate intermediates. The data suggest a more favorable metabolism for fructose than for glucose in these diseases. The authors conclude that "fructose may be the sugar of choice in diseases involving glucose-impaired metabolism."

Further evidence of a difference in the metabolism of fructose and glucose was obtained by Wyshak & Chaikoff (9) in studies of carbohydrate utilization in livers of fasted (48 to 72 hr.) rats. The conversion of radioactive glucose to CO_2 , fatty acids, and glycogen was depressed (50 per cent) while the conversion of radioactive fructose remained normal in the fasted rat. The point of inhibition was established as an interference with the glucokinase

reaction with a secondary block in lipogenesis. Recovery occurred upon administration of glucose.

PROTEINS

The metabolism and physiology of the D-amino acids have been reviewed by Berg (10). Other reviews on various aspects of protein and amino acids in nutrition have appeared during the past year by Allison (11), Flodin (12), and Albanese (13).

A considerable amount of research has been reported on the biological quality and availability of various proteins and protein supplements. The effect of heat treatment on vegetable proteins has been studied by several workers. Further purification of the toxic protein, soyin, of raw soybeans by Liener & Rose (14) showed that defatted soybean meal flours contained about 3 per cent soyin. Anorexia was found by Liener (15) to account for 50 per cent of the growth retardation (an effect which did not respond to the addition of crude trypsin) which resulted when soyin was included in a ration fed to rats. From the results of liver xanthine oxidase studies, Liener & Wada (16) concluded that heat treatment of soybean flour increased the availability of the methionine which in turn could be used for the synthesis of enzyme protein; however, the poor growth on raw soybean flour was not entirely a result of impairment in the availability of methionine. Another study of similar nature was made by Carroll and co-workers (17). Heat treatment of soybean meal resulted in an improved absorption of nitrogen and amino acids from the small intestine of the rat. Heat treatment or aureomycin supplementation of raw or heated meal increased the amount of nitrogen absorbed while dietary supplements of methionine were ineffective. Similar results were obtained by Becker and co-workers (18). Hill and co-workers (19) did not find the lack of amino acids to be the cause of growth retardation of chicks fed raw soybean meal. The effect of supplements on the growth of animals fed raw soybean meal in these studies is difficult to interpret in the light of the known content of both a toxic protein and a digestive enzyme inhibitor. An effect of trichloroethylene-extracted unheated soybean oil meal on the blood clotting time of chicks which did not respond to vitamin K supplements has been observed by Balloun & Johnson (20).

Heat treatment has been shown by Sure (21) to affect the protein efficiency of cottonseed meal and flour. Moderate heat treatment increased the protein efficiency whereas more drastic treatment decreased its efficiency. This was found to be associated with the unavailability of lysine and methionine. Renner and co-workers (22) found a similar differential effect of heat treatment on the availability of lysine, arginine, and tryptophan in sunflower meal. Schroeder *et al.* (23) reported that autoclaving whole raw milk at 10 to 15 lbs. pressure for 30 min. had no detrimental effect on its digestibility or biological value. However, changes in the properties of milk subjected to rather mild treatment have been observed which may affect its biological value and digestibility under certain conditions. Ramsdale *et al.*

(24) demonstrated that the albumin and globulin were denatured by heat and thus rendered coagulable by acids, salts, and rennin. Zweig & Block (25) showed that the heating of milk (71° to 77° C.) caused an initial rise in titratable sulfhydryl groups which was quickly followed by a decrease. The exposure of milk stored in ordinary glass bottles to light for $\frac{1}{2}$ hr. or more was found by Patton & Josephson (26) to result in the development of activated flavors which had their origin in methionine and were dependent upon the presence of riboflavin. The ascorbic acid and riboflavin were destroyed as the activated flavors developed. By means of labeled lactose-1-C¹⁴, Patton & Flipse (27) were able to demonstrate that the amount of radioactivity found in the protein was related to the heat treatment and the degree of browning in milk. The binding of lactose by the protein occurred prior to the development of color.

Recent studies on the protein and amino acid requirements of young pigs have shown that during the early days of life the protein requirement of the baby pig is very high. Reber and co-workers (28), who used growth rate, feed utilization, nitrogen retention, and certain blood components as criteria, found values of 41 per cent for the very young pig and 20 per cent at eight weeks of age. Sewell and co-workers (29) found the requirement to be approximately 24 to 28 per cent during the fastest growing period with the most efficient feed utilization occurring at the 32 per cent level. The studies of Sewell and co-workers (30) with suckling pigs fed semisynthetic milk diets have indicated a requirement of threonine of 0.9 per cent which was equivalent to 3.5 per cent of the dietary protein. Jackson and co-workers (31) found the valine requirement of the pig to be 0.4 per cent of the ration.

The adequacy of various single proteins or commercially available protein concentrates to meet growth requirement of several species has been studied by several laboratories. Mitchell and co-workers (32) reported much needed information on the nutritional value of corn protein, a problem which has been accentuated by the development and use of hybrid corns of variable protein content. The proportion of zein in the total protein of the corn increased linearly with the increase in protein content up to 14 per cent. Concurrently, the proportions of tryptophan and lysine in the total protein decreased with increasing protein content of the corn. It is not surprising therefore that the biological value of the protein decreased with increased protein content. The addition of lysine and tryptophan raised the biological value of the corn protein to the approximate value of meat protein. Bloss (33) found that a ration of ground corn, meat, and bone scraps required the addition of 0.06 per cent DL-tryptophan for pigs fed this ration to show growth gains comparable to that of controls fed soybean oil meal. Aureomycin was not effective in counteracting the tryptophan deficiency of the basal.

A study of the amino acid content of molasses and cane juice was made by Kowkabany and co-workers (34) who reported the presence of asparagine, aspartic acid, glutamine, glutamic acid, glycine, alanine, valine, leucine, serine, tyrosine, and 3-amino mutaric acid, as well as small amounts of thre-

onine, phenylalanine, choline, and arginine. The use of supplements to single protein diets has been studied by Sure (35) who found that the addition of lysine, valine, threonine, and an extract of condensed fish solubles increased the biological value and efficiency of utilization of milled wheat flour. A similar study of single protein diets was made by Albanese (36) who found that feeding wheat gluten to infants resulted in a depression in nitrogen retention accompanied by an increase in blood plasma protein. Supplementation with lysine restored the values of these criteria to within normal range. In a series of studies of the effect of wheat gluten (or rye protein) on the steatorrhea of humans, van de Kamar *et al.* (37) observed that wheat gluten through its gliadin component increased the fecal fat excretion of these patients.

Other factors such as mineral or vitamin levels, rancid fat, starvation, or hormones in the diet have been shown to modify the utilization and metabolism of dietary protein. Benton and co-workers (38) continued their studies of the effect of vitamin B₆ upon the utilization of certain amino acids and found that B₆ was essential for the utilization of glutamic acid, lysine, methionine, and histidine as well as cystine, glycine, tryptophan, and alanine, previously reported. B₆ deficiency resulted in an increased formation of urea from several of the amino acids. An interesting observation was reported by Axelrod (39) who found that antibody production depended upon the integrity of the diet with respect to members of the B group and certain amino acids. The evidence indicated that the vitamins pyridoxine, pantothenic acid, and pteroylglutamic acid were specifically concerned, and riboflavin, thiamine, biotin, vitamin A and niacin, and tryptophan were moderately effective. Greenberg & Frazer (40) have shown that rancid fat in the diet increased the protein requirement. A protein balance was reached when the diet contained 30 per cent protein but growth was still inferior to that of control rats not fed rancid fats. Cortisone reduced growth; an observation also made by Clark (41) who studied the effect of cortisone on protein synthesis. An increased urinary excretion of nitrogen was observed. Clark concluded that cortisone impaired protein synthesis by interfering with the formation of new protein.

Wainio and co-workers (42) studied the effect of starvation and protein depreciation on the oxidative enzymes of the liver of the rat. A reduction of protein either by a low protein diet or by starvation, reduced the xanthine oxidase, cytochrome-*c* oxidase, diphosphopyridine nucleotide-cytochrome-*c* reductase, and uricase. Low protein has no effect on cytochrome oxidase but in all others there was a marked reduction in the activity. The author concludes, "The general decrease in activity per unit of nitrogen indicates that the enzyme proteins were lost more rapidly than the loss of total protein. The cytochrome oxidase protein was conserved."

The technique of protein depletion and repletion has been used by two groups of investigators to demonstrate the effect of minerals on protein catabolism and anabolism. Menaker & Kleiner (43) reported a strikingly unfavorable effect upon protein regeneration when the repletion diet was low

in magnesium. The addition of calcium chloride or sodium chloride or both exerted a favorable influence upon protein regeneration as measured by growth (rat). The detrimental effect of low potassium repletion diets was reported by Cannon *et al.* (44) in producing severe degenerative lesions in various tissues. The ratio of sodium to potassium has been found by Burns *et al.* (45) to be as important as the actual dietary requirement level as determined by growth response. The dietary levels in this study included the toxic extremes. Frost & Sande (46) found that supplements of amino acids did not prevent the complete failure and death of potassium-deprived animals. Although many investigators have used extreme conditions and dietary levels in demonstrating the growth-depressing and toxic effects of mineral imbalances, a partial explanation in the depletion-repletion studies may be inferred from the work of Hegsted *et al.* (47). They found a marked increase in muscle extracellular fluid space in protein-depleted animals at low environmental temperatures which was correlated with the severity of the depletion. Low environmental temperatures are known to increase the vitamin requirements. Von Korff (48) is of the opinion that the conversion of acetate to the high energy intermediate acetyl CoA can be classified with those enzymatic processes shown to be stimulated by potassium ion and inhibited by the sodium ion. Methionine injections have been observed by Ely and co-workers (49) to prevent the symptoms of toxemia resulting from injecting toxic levels of radioactive cobalt, but they did not alter the anatomical distribution of the Cobalt.

Evidence has been accumulated to show that the proper spacing interval in the feeding of amino acids regulates the growth rate of the experimental animals. Eggert and co-workers (50) applied the spacing interval principle to whole protein supplements in rations of swine. They found that growth was rapid and nitrogen utilization comparable to results with normal interval feeding practices of 24 hr. or less, but at longer intervals (36 to 48 hr.) a decrease in nitrogen retention of approximately 7 to 14 per cent was observed.

Data on the effect of antibiotics in improving the growth of animals fed low protein diets and in affecting the digestibility of the ration have been presented by several investigators. Wilson *et al.* (51) found that the addition of aureomycin to the ration of weanling pigs stimulated growth at the low protein levels but not when the diet contained 20 per cent protein. Similar results were obtained with penicillin by Saxena and co-workers (52). Terramycin was observed by Huang *et al.* (53) to give greater growth response than alfalfa, which was variable, cobalamin (vitamin B₁₂) which was negative, or soil which gave some growth improvement, when these supplements were added to a purified ration fed to growing pigs. Rusoff *et al.* (54) obtained improved growth when young dairy calves were fed an aureomycin supplemented ration and were unable to correlate any response in the intestinal microflora which was attributable to the aureomycin. Chance and co-workers (55) found that when 0.5 gm. of aureomycin was fed the total bacterial count of rumen ingesta and feces of fistulated steers increased; the

streptococci population decreased and the coliform population increased. In other studies Chance *et al.* (56) observed that the rate of removal of dry matter, crude fiber, crude protein, and nitrogen-free extract in cattle was highest when 0.5 gm. of aureomycin was fed and that a higher level (1 gm.) slightly depressed digestive activity. Direct evidence of an effect on amino acid synthesis was not found by this group of workers (57), but a decrease in riboflavin concentration and nicotinic acid synthesis was noted.

Studies on the requirement and metabolism of individual amino acids showed that the protein requirement is dependent not only upon the absolute amount but also on the relative proportions of amino acids. Womack and co-workers (58) studied the influence of certain nonessential amino acids on the maintenance requirement of the adult male rat for methionine, isoleucine, and threonine. Increasing the dietary cystine decreased the methionine requirement. The isoleucine and threonine requirements of 11 and 10 mg. respectively, per rat per day, were not affected by nonessential amino acids. White *et al.* (59) presented evidence that D-valine could replace the natural L-form, but the rats grew half as well as those fed the L-form. It is believed that D-valine was deaminated and reaminated in the liver to the L-form. In a study of the synthesis of glycine and serine by the rat, Arnstein & Neuberger (60) concluded that the rather constant glycine content of tissues was maintained by regulation of the rate of degradation rather than the rate of synthesis and suggested that the likely pathway of endogenous glycine metabolism was through serine. The results of anthranilic acid metabolism studies by Mason (61) have indicated a derangement of tryptophan metabolism in riboflavin-deficient rats. A vitamin-amino acid relationship was studied by Henderson, Koeppe & Zimmerman (62) who found that the addition of threonine to a diet which contained a marginal dietary level of tryptophan resulted in a growth depression which could be counteracted by supplements of niacin. These studies highlight several years of study on the limiting amino acids in low protein diets and the tryptophan-niacin dietary interrelationships. Similar findings were observed by several investigators (see fatty liver section). Studies on wound healing by Williamson & Fromm (63) indicate that cystine has a greater stimulatory effect in regenerating wounds than methionine, and the rate of healing can be correlated with the cystine content of the wound. Findlay (64) observed that cobalamin increased the tensile strength of wounds during the early phases of healing in rats fed a balanced diet but not on a protein-depleted diet.

Several studies have been made on the dietary amino acids and other metabolites in the blood. Investigators have attempted to determine the origin of the ergothioneine of blood. Heath and co-workers (65) found that the S³⁵ of sulfate sulfur or thiohistidine did not appear in ergothioneine whereas that of the dietary methionine did. Ergothioneine also appeared in the seminal plasma of the boar. When the synthetic ergothioneine containing S³⁵ was fed to rats [Heath (66)], the compound appeared in the blood and tissues of the animal. When Melville *et al.* (67) fed C¹⁴ labeled methionine to

chicks and rats, they did not find any activity in the blood ergothioneine. Baldridge & Lewis (68) found that the blood level of ergothioneine of rabbits was more readily altered by different diets than that of rats. An oats-cabbage diet was found to be an ergothioneinogenic diet for rabbits. No ergothioneine could be detected in the diet itself. The dietary effect of this compound is interesting in the light of earlier work by Mann & Leone (69) who showed that ergothioneine was concentrated only in the red blood cells (pig) and was found in the seminal fluids of the boar in amounts of 29 to 256 mg. per 100 ml. From earlier data they suggested that the function of ergothioneine was to control —SH inhibitors as demonstrated in sperm and muscle glycogenolysis. This compound afforded protection against the oxidase activity of cupric ions and hydrogen peroxide. The glutathione content of red blood cells was observed by Mortenson (70) to follow closely the supplementation and withdrawal of methionine from the diet.

The interest in nonprotein nitrogen as a source of protein for ruminants continues. An excellent review of this subject was presented by Reid (71). Urea diets in which 40 per cent of the total nitrogen of the ration was furnished by urea were used by Lofgren, Weir & Wilson (72) in studies on the effect of inorganic sulfur on wool production and growth of sheep. The addition of sodium sulfate (0.2 per cent) did not increase the rate of body weight gain or wool growth. These findings are in contrast to reports from the Illinois Experiment Station. A question is raised concerning the disappearance of the sulfate from the paunch contents of sheep since these authors found that only a trace of inorganic sulfur could be detected 3 to 5 hr. after feeding. Tillman & Swift (73) studied the effect of different nitrogen-containing compounds, ammoniated condensed distillers solubles, ammoniated cane molasses, and urea when these compounds furnished a part of the nitrogen in the ration which was otherwise composed of natural feedstuffs. A decrease in digestibility of all ration constituents except the ether extract and a lower nitrogen storage was found when ammoniated products were used. In contrast, urea-containing, as well as soybean rations, gave excellent and comparable results. These data suggest caution in the use of ammoniated feeds or ammonium salts in the feeding of ruminants, particularly sheep. Urea was found by Bouchaert & Oyaert (74) to be toxic for sheep under certain experimental conditions. When urea was injected directly into the rumen, the mortality rate of animals fed a straw diet was very high in comparison with results on alfalfa-fed sheep. These workers maintain that urea is toxic in the sheep because it is converted to ammonia which penetrates the rumen wall causing rumen paralysis and hence death.

As a result of studies with calves Agrawala *et al.* (75) estimate that calves synthesized 33 to 109 gm. of protein daily from urea fed as the sole source of nitrogen in a purified ration. Duncan and co-workers (76) found that the rumen microorganisms of fistulated calves fed urea as the sole source of nitrogen could utilize urea nitrogen to synthesize all of the ten essential amino acids with the one exception of histidine. The amino acid pattern of

the ingesta was reported to be similar to that of calves fed natural diets with protein.

Quantitative evidence was obtained by Agrawala *et al.* (77) which indicated that pantothenic acid synthesis in the bovine rumen when urea was used as the only source of N was reduced 50 per cent (6 hr. post-feeding). These results were checked against rumen synthesis of similar animals given a normal ration. There was no dietary effect on the rate of synthesis of riboflavin or niacin.

Prescott (78) reported *in vitro* studies of the effect of antibiotics on the utilization of nonprotein nitrogen. Test material was obtained from slaughter steers by expressing the rumen liquid (possibly not a true incubation sample) from the ingesta and adding nonprotein nitrogen compounds to the incubation juice. Administration of high levels of aureomycin, terramycin, and penicillin in these *in vitro* trials indicated that these compounds are capable of reducing the utilization of nonprotein nitrogen. This confirms the *in vivo* work of Huffman's group (56) who showed that high levels of antibiotics interfered with the utilization of nitrogen. Thus the majority of data indicate that urea can be fed to ruminants as a source of nitrogen for protein synthesis in the rumen. It is also clear that certain precautionary steps must be taken to insure success. Since the conversion of nitrogen to urea involves energy, an energy supplement or source would be indicated in the use of urea to replace protein in the ration of ruminants. Animals in a semi-starved condition as a result of either poor feed, or low feed intake, or marginal dietary constituents, would not be likely to respond favorably to urea feeding. This undoubtedly explains why some of the research workers have failed to obtain positive results from feeding urea to ruminants.

FATS

Several interesting observations on the relationship of fats to diet and nutrition have been made during the past year. When Swank & Cullen (79) administered large fat meals to the golden hamster they observed in the cheek pouch circulation increased adhesiveness and aggregation of the red blood corpuscles accompanied by a slowing and an occasional cessation of flow of the blood. These changes occurred after the peak of the lipemia had passed, and developed their maxima as the lipemia cleared. The blood returned to normal following the clearing of the lipemia and suspension stability was regained. This study is suggestive as far as the intake of fat is concerned and would indicate that high fat intakes should be given in moderate amounts, evenly distributed over a rather long period of time, rather than at a high ingestion level in a short period. A stimulating report was presented by Kramar & Levine (80). Because these authors had noted a variation in capillary resistance between clinical cases subsisting largely on vegetable fat and those subsisting on animal fats, they postulated that a substance in these fats might be capable of damaging the capillaries themselves, interfere with the resistance, or that a substance might be present in the dietary fat that

was required for the structural integrity of the capillary bed. Experiments with rats fed fat-deficient diets showed that the reduced capillary resistance correlated well with the flattening out of the weight curve, but it was measurable before other manifestations of a fat deficiency occurred. Low capillary resistance of animals fed synthetic diets devoid of fatty acids or containing methyl esters was found to be restored by feeding small amounts of linseed, cottonseed, or pure linoleic acid.

Obesity is a serious disease affecting the human. Lyon and co-workers (81) found that they could develop obesity in strain $C_{57}H$ mice by feeding a high fat diet containing casein 30 per cent, dextrin 15 per cent, salts plus vitamin mix 5 per cent, and 50 per cent fat. Strain $C_{57}H$ was resistant to the development of obesity on this ration. It has been an accepted fact that animals eat to meet their caloric requirements, but these authors found that both strains of mice ($C_{57}H$ and $C_{3}H$) ate more calories when they were given the high fat diet than when they were given the rations containing 5 per cent fat. Observations on the oxygen consumption showed that strain $C_{57}H$ oxidized more calories with a greater consumption of oxygen; strain $C_{3}H$ was unable to eliminate the additional calories in this manner and therefore became obese. While it is true that both strains of mice ate 50 per cent more calories on the high fat diet than they did on the control diet, it is interesting to point out that daily food consumption was cut from 25 to 30 per cent (total food consumption) over that of the control rats. The reduced food intake would lower the protein consumed by some 30 per cent and while superficially this would not seem to be significant, the drop in actual protein intake in the presence of a very high fat diet might affect absorption to the point where protein became the critical constituent. Furthermore, the high fat ration contained too little carbohydrate for optimum utilization of fat. This would seem to be the case in light of the work of Masoro and co-workers (82) who demonstrated that high protein or high fat diets depressed the utilization of glucose by liver slices. The effect of reduced protein ingestion is further substantiated by the report of Campbell *et al.* (83) who found that the addition of 15 per cent fat to a ration containing 3 to 4 per cent fat reduced the daily food intake of young growing dogs sufficiently to affect the amount of protein ingested. This retardation in growth could be regained by the addition of methionine and lysine to the ration.

Beare *et al.* (84) showed that a B_6 deficiency in young rats caused a decrease in the total fatty acids, even when corn oil was fed, and small decreases in water and protein. The effect of B_1 and B_{12} was similar to that of B_6 but to a lesser degree.

The prominence of coronary disease and arteriosclerosis as a major cause of debilitation and death in man prompts an active interest in research on cholesterol and fat. Recent observations on the incidence of this disease in humans by Henschen (85) showed that people of North Sweden whose diet consisted primarily of a lacto-vegetable and fish, were slim and had a lower incidence of the disease than people of Stockholm who were generally more

obese. Walker *et al.* (86) found that caloric restriction in humans leads to a significant reduction of the serum levels of lipids and lipoproteins. A general cholesterol intake (two eggs daily) made no significant difference in the results. Keys (87) has recently reviewed his studies in which similar results were obtained. Betaine was found by Morrison (88) to be the best tolerated and most effective lipotropic agent among many which caused a significant decrease in cholesterol and an increase in phospholipid levels of serum of patients which had been fed a low-fat low-cholesterol diet. Glass *et al.* (89) and Elbert (90) observed little or no effect on the total lipid level of serum when estrogen was administered to patients. Elbert noted that this treatment affected the partition of lipids with a depression in the cholesterol and elevation of the phospholipid levels.

It is well known that all species so far tested destroy cholesterol when it is ingested in large amounts. In order to produce cholesterolemia and atherosclerosis experimentally, it is necessary to feed large amounts of cholesterol and in some species, thyroid-inhibiting agents as well. Gould *et al.* (91) studied the effect of dietary cholesterol on the endogenous cholesterol synthesis in pups as indicated by the incorporation of C^{14} from labeled acetate. The rate of cholesterol synthesis which was greater in the liver than the intestinal mucosa, was decreased when cholesterol was fed. Similar findings were observed by Frantz *et al.* (92) in the rat in which the thyroid was inhibited by I^{131} . High cholesterol feeding alone produced no significant rise in serum cholesterol but resulted in a two-fold increase in liver cholesterol. After inhibition of the thyroid with I^{131} , an 80 per cent increase in serum cholesterol was observed, and the liver cholesterol was trebled. In other experiments by Swell & Flick (93) with rats the results suggested that absorption of cholesterol was less when a high percentage of saturated fatty acid was present in the diet than when the fats were unsaturated.

Altschul (94) has recently reported the interesting observation that if rabbits were fed cholesterol and exposed at intervals to ultraviolet irradiation for approximately 3 months, a marked or complete inhibition of cholesterol effects was observed. The investigator postulated that cholesterol changed to lumicholesterol which was less effective or that the irradiation resulted in the production of protective substances. Nelson *et al.* (95) found that the inclusion of aureomycin in the diet of rabbits resulted in a greater incidence and severity of atherosclerosis with characteristic effects on blood serum. A species difference in the effect of pyridoxine was observed by McFarland (96). Although pyridoxine deficiency aids in the production of atherosclerosis in monkeys, the addition of high levels of this vitamin to chick diets had no alleviating effect and in many instances tended to increase the severity of the disease. The effect of intermittent feeding of cholesterol was studied by Rodhard *et al.* (97). Cockerels fed a mash and cholesterol-mash diet alternately showed less atherosclerosis and lower plasma cholesterol. When the cockerels were starved or fed noncholesterol, oil rich diets alternately with the cholesterol-mash diet, the high cholesterol serum level and atherosclerosis

damage noted in a continuous cholesterol feeding was observed. Gordon *et al.* (98) and Peterson *et al.* (99) studied factors which decreased the cholesterolemia in chicks. Feeding of brain residue (98) reduced the serum cholesterol and markedly reduced the level of high and low density lipoproteins. Soya sterols (99) prevented the cholesterolemia in ratios of 2:1 or 3:1 (sterol: cholesterol) in the diet. Esterification of the sterols destroyed this effect. The dietary fat effect in maintaining the cholesterolemia was also observed. Both groups of investigators postulated a decreased cholesterol absorption as part of the mechanism of these factors. The production of atherosclerosis in *Cebus* monkeys was reported by Mann *et al.* (100) as a result of feeding diets high in cholesterol (5 per cent) and low in sulfur amino acids. The lesions could be largely prevented by feeding DL-methionine and partially prevented by L-cystine. A new method of detecting the presence of S₁ 2-30 lipoprotein fraction was reported by Baker & Ogden (101). The serum of rabbits immunized with this fraction obtained from cholesterol-thiouracil-fed dogs showed an abnormally increased concentration of these serum lipoproteins in atherosclerotic dogs and man. Normal sera from dogs and man showed much less or no reactivity.

Several *in vivo* and *in vitro* studies have been reported on the relationship of squalene to cholesterol [Tompkins (102); Langdon & Bloch (103, 104, 105)]. Squalene was shown to be synthesized in the rat and was a normal constituent in liver. The biosynthesis of cholesterol from squalene is very "stero specific" in that the C¹⁴ of biologically labeled squalene was incorporated into cholesterol whereas that of synthetic squalene was not; natural squalene decreased the incorporation of C¹⁴ from acetate into cholesterol whereas the synthetic squalene did not. It is believed that squalene is preferentially incorporated into cholesterol to the exclusion of the C¹⁴ from acetate. When squalene was fed at a level of 3 per cent in the ration to rabbits by Kritchevsky *et al.* (106) no atherosclerosis was observed in contrast to the numerous lesions observed in animals fed a similar level of cholesterol for the same period of time.

FATTY LIVERS

Explorations of the cause of nutritionally produced "fatty livers" have been aimed at two problems: (a) the role of choline, and (b) the role of low protein or amino acid imbalance diets containing choline. "Fatty livers" as used by research workers in the field of nutrition denotes increased comparative liver fat content as measured by solvent extraction. The term further implies hepatic cell damage in current nutritional language which is all too frequently without demonstrable proof.

The recent studies by Koch-Weser and co-workers (107) in which low and adequate protein diets were used has permitted a histopathologic differentiation of the effect of choline on cell damage and metamorphosis of fat in the rat liver. Administration of choline by stomach tube to rats which had been

on a moderate protein, low fat, choline-deficient diet for four weeks resulted in almost complete removal of the liver fat in 48 hr. as determined by histopathologic examination and chemical analysis of the liver. No cell damage was seen as the result of this choline deficiency. However, the feeding of a low protein, high fat diet with adequate choline for a similar length of time resulted in a dual effect, fatty metamorphosis and cell damage. Again the administration of choline effected a reduction in hepatic fat within 48 hr., but it had no effect upon the cell damage. The failure of choline to benefit cells at the degenerative stage was pointed out last year in Gyorgi's review.

The studies of Bligh (108) may throw some light on the subject. The rapid disappearance of injected choline from the blood was confirmed, and in addition it was found that the removal of both kidneys and liver together reduced the rate of disappearance but did not halt it. Artom (109) found that the labeled CO_2 released from liver slices of choline-deficient rats was low when incubated with labeled stearate and that of animals injected with choline $1\frac{1}{2}$ hr. before sacrifice was high. Further studies by this investigator (110) indicate that an active factor was firmly bound in cytoplasmic granules as a water-insoluble combination which was formed *in vivo* but not *in vitro*. Choline, betaine, betainealdehyde, or phosphoryl choline were inactive *in vitro*. On the other hand, studies of choline oxidase activities by Hummoller & Zimmerman (111) showed that injection of choline or betaine 2 hr. before sacrifice did not alter the results with this system with choline deficient animals.

Recently Hawk & Elvehjem (112) used a 9 per cent casein, high fat diet and obtained lipotropic activity with cobalamin in the presence of .02% dietary choline, but not in its absence. The activity of cobalamin was explained on the basis of its sparing action upon choline. Lipotropic activity of liver extract when a high fat diet was fed has been shown by McCormick *et al.* (113) to be independent of its choline, inositol, folic acid, or cobalamin content. In the light of recent failures of the known lipotropic factors to be effective when the protein intake is limited further study of this extract would be of interest since Drill & Hall (114) described cell damage as well as liver fat accumulation.

An interesting report of "serous hepatitis" in Jamaican children has appeared and has been associated by Hill *et al.* (115) with a low dietary protein intake and possibly with toxic factors.

Studies on the limiting amino acids which affected growth response of animals fed low protein diets led Singal *et al.* (116) to the observation that these animals showed increased accumulation of liver fat which was prevented by the addition of threonine to the diet. Fat accumulation and factors which affect it have been further studied by several groups of investigators during the past year [Litwack *et al.* (117); Singal *et al.* (118, 119); Harper *et al.* (120); Sauberlich (121); and others]. In all these studies the basal diets contained choline and in most instances the other common lipo-

tropic agents as well. Confusion exists in some of these reports because of the dual studies of the niacin-tryptophan relationship and the lipotropic effect of threonine. The niacin-tryptophan relationship to the threonine content of the diet has been explained by Koeppe & Henderson (122) and confirmed by the others. Singal *et al.* (118) also showed that threonine prevented the liver fat accumulation in animals pair-fed high or low fat diets. Litwack *et al.* (117) found that the type of carbohydrate affected the growth response of animals fed low protein diets, an effect also noted by Harper & Katayama (123). The critical dietary protein (casein) level was shown by Harper *et al.* (120) to be 9 per cent since 11 per cent prevented the fat accumulation and by Saubertlich (121) who demonstrated that the lipotropic effect of threonine and other factors was ineffective when 7 per cent protein level was used.

A threonine-deficient diet which supplied all other of the essential amino acids produced liver fat accumulation in the rat [Dick *et al.* (124)] unless non-essential amino acids equivalent to 9 per cent casein were also included (118). None of the essential or nonessential amino acids tested have replaced threonine in preventing liver fat accumulation although a few appear to reduce the level of threonine required (120). These studies indicate the necessity for rigid control of the diet in experimental procedures which use a very limited dietary protein level. In this connection it is of interest to note the reports of Kandutsch & Baumann (125) and of Rombouts (126) on the effect of various dietary constituents on the destruction of B vitamins in synthetic diets. Singal *et al.* (119) found that threonine- (or lysine) deficiency depressed the synthesis of phospholipids and nucleoprotein phosphorus fractions in the liver.

The recent findings of Morgan & Lewis (127) are of interest since they found that fatty livers do not occur in rats fed choline-deficient diets if a simultaneous deficiency of pantothenic acid is produced. This brings another factor into the chain of events leading to "fatty livers." Changes in the pituitary-adrenal hormonal activity are implicated in these studies. Klein & Lipmann (128) also found the liver cholesterol and fat levels to be low in pantothenic acid-deficient animals. Both reports indicate that the effect of pantothenic acid is dependent upon its effect on coenzyme A metabolism.

Muscle dystrophy.—The feeding of cod liver oil to calves under practical conditions produced muscle dystrophy, damage to heart muscle with gray-ing, and frequently heart failure according to Blaxter *et al.* (129). Blood serum tocopherol content of affected cattle was 60 μ g. per cent as compared to values of 270 μ g. per cent for normal cattle. These results are similar to those reported by Gulickson and co-workers (130). A new antivitamin, tri-cresyl-phosphate, was reported by Myers *et al.* (131) which may help in the study of the relationship of α -tocopherol to muscle dystrophy. Chronic doses fed with wheat germ oil to male rats produced an irreversible sterility in two to three months and paralysis in ten months. Tri-phenyl-phosphate also produced similar effects.

ANTIBIOTICS IN NUTRITION

Animal response to dietary antibiotics is best demonstrated with animals kept under unfavorable environmental or restrictive dietary conditions. Why marginal intakes of any dietary factor will cause animals to respond favorably to the addition of antibiotics is not understood. The failure of antibiotics to improve upon an adequate diet, particularly with sufficient protein content, has been repeatedly shown (51, 53, 132, 142).

There are differences in the effects of the different antibiotics as well as species differences in the response. Chicks or poultry require less accentuation of the unfavorable factors in order to give a measurable response in growth. Antibiotics give a measurable and economic growth response in broilers and therefore have a practical dietary application to the broiler industry.

Braude, Kon & Porter (133) have written an excellent review on the subject of antibiotics in nutrition. The alteration of bacterial populations of the feces of rats has been studied by Guzman-Garcia *et al.* (134) with penicillin, by Johansson and co-workers (135) with aureomycin, and in the cecum of the chick by Anderson *et al.* (136). The influence of feeding marginal diets has been studied by these authors and by Guggenheim *et al.* (137). Guzman-Garcia (135) demonstrated that the diet of rats must be less adequate comparatively than that of chicks to obtain significant results.

In an attempt to counteract the antivitamin activity of toxic levels of aminopterin, Sauberlich (138) found that leucovorin (CF), 10 mg. per kg. of diet, would prevent the inhibition but ascorbic acid, aureomycin, or penicillin were without effect. Chow and co-workers (139) who used aureomycin, bacitracin, streptomycin, or penicillin observed that these antibiotics differed in their effects as measured by the fecal content of cobalamin. Several studies have been made during the past year in the field of poultry nutrition [Anderson & co-workers (136); Waibel, Cravens & Baumann (140); Gerard *et al.* (141); Atkinson (142)]. Anderson and co-workers found that an atypical strain of *Escherichia coli* stimulated weight gain in young chicks equal to that of dietary penicillin. Certain micrococci depressed growth in the absence of penicillin. Three antibiotics, penicillin, aureomycin, and a mixture of antibiotics markedly decreased the growth of chicks fed diets containing limited amounts of thiamine. Waibel *et al.* (140) noted that antibiotics had no effect on growth if the limiting amount of thiamine was injected rather than fed. Atkinson (142) observed that penicillin and bacitracin promoted a slight increase in growth of poults but antibiotics did not always stimulate growth. The soybean oil meal, corn, vitamins, and mineral ration used is typical of the all plant rations commonly used to demonstrate antibiotic effects.

Much work has been done on the relationship of antibiotics to growth of pigs, feed utilization, and quality of the carcass; [Huang & McCabe (143); Lehrer and co-workers (144); Perry *et al.* (145); Wallace *et al.* (146); Luecke

et al. (147)]. In general the addition of antibiotics to all plant rations improved growth and increased feed utilization, but the effects on dressing percentage and carcass quality were conflicting. Luecke *et al.* (147) found that aureomycin (5 mg./lb. of feed) did not spare pantothenic acid. On the other hand under the experimental conditions of the studies of Catron and co-workers (148) a response was obtained with the addition of aureomycin (10 mg./lb. of ration) to a ration which was also improved by supplementation with pantothenic acid.

Considerable progress has been made in studying the effect of antibiotics on young cattle, but the results of these experiments were not always favorable [Knodt & Ross (149); Bloom & Knodt (150); Bartley & co-workers (151); McKay, Riddell & Fitzsimmons (152); Bartley & co-workers (153)]. Incorporation of aureomycin (20 mg./calf/day) in milk replacements and calf starters was without benefit on weight gain and feed consumption in the studies of Knodt *et al.* (149, 150). Bartley *et al.* on the other hand found that 15 mg./100 lbs. body weight per day increased growth significantly. The feeding of penicillin (Knodt) resulted in decreased growth rates.

Data are beginning to appear in the medical literature on the deleterious effect of prolonged administration of antibiotics. They suggest that long term studies using experimental animals would be of value. It is conceivable that what is now considered a failure of antibiotics to "spare" vitamins might be found in long term studies to result in a deleterious effect in view of the demonstrated increases in antibiotic-resistant organisms in the gastrointestinal tract. Klotz *et al.* (154) found that the prolonged administration of antibiotics resulted in proctitis and colitis, intestinal disturbances more serious than the original disease. Slanetz (155) observed that the initial effect of antibiotics was an increase in antibody titer but continued administration resulted in an interference with antibody formation.

REPRODUCTION

The determination of the cause of reproduction and lactation failures remains a challenge to investigators in the field of nutrition. Palsson & Grimsson (156) report that young lambs from ewes pastured on seaweed developed ataxia with cerebral lesions. Blood copper values were 0.2 p.p.m. while liver concentrations were less than 7 p.p.m. The disease could be prevented by copper supplements to the diets of the pregnant ewes. The effect of various dietary protein levels on gestation has been studied by Nelson & Evans (157) who found that 5 per cent protein was the critical level for obtaining full-term gestation. Doubling the vitamin supplements for the rats fed lower dietary protein levels decreased the incidence of fetal resorptions. Strikingly, similar results on low protein diets were obtained by Curtiss (158) who also studied the effects of choline deficient and supplemented diets. Campbell and co-workers (159, 160) have observed a species difference in the ribonucleic acid (RNA) content of liver cells of pregnant and nonpregnant animals and found a correlation of the increase in RNA with the presence

of viable placenta. Studies on the lactation phase of reproduction by Dryden *et al.* (161) showed that cobalamin-deficient diets interfered with lactation performance, results which confirm earlier work of Nell & Phillips (162). The observations of Dryden *et al.* (161) indicated that the decreased quantity of milk produced by cobalamin-deficient mothers was a primary factor in explaining the lower weight gains of the young. Collins and co-workers (163) found the colostrum of goats to be a richer source of cobalamin than milk. Both were from does fed trace mineralized diets. An interesting observation on the effect of trace minerals was observed by Nishimura (164) who found that newborn mice without colostrum developed a series of symptoms when transferred to foster mothers in late stages of lactation which were cured by the oral administration of inorganic Zn. A difference in the vitamin E, cobalamin and trace mineral requirements of two strains of mice is indicated by the studies of Lee *et al.* (165). Schultze (166) found that the lactation failure of rats fed diets containing purified soybean proteins was corrected by crude protein from defatted nervous tissue and partly by vitamin-free casein but not by folic acid or grass juice concentrate.

Anos & Finerty (167) reinvestigated the effect of a fat-free diet on growing female rats with special reference to the endocrine glands. Organ weights reported for the thyroid and adrenals were smaller while those for the kidneys, liver, and heart were larger in the fat-free animals than in those receiving normal dietary fat. All of the experimental animals (low fat diet) developed dermatitis of the tail, feet, and loss of hair over the back. Estrus became irregular after 18 to 20 weeks on the diet. The authors suggest that a fat deficiency causes a reduced secretion of the luteinizing hormone from the acidophile cells (pituitary) which results in a reduction of ovarian stimulatory effects, and hence, the consequent disturbance of the estrus cycle. Biosynthesis of milk fat in the mammary gland of rabbits was studied by means of C^{14} by Popjak, Hunter & French (168). Both short and long-chain fatty acids were synthesized from acetate and from glucose in the lactating gland of the rabbit.

The reproduction studies with larger animals have been concerned primarily with attempts to formulate semi-synthetic milks which would take the place of colostrum. Ronning and co-workers (169) have studied the carotene requirements for successful reproduction in Guernsey cows over an eight year period in which 72 gestation periods were observed. It appeared that the safe minimum level of carotene intake (supplied from prairie hay) was 90 μ g. per lb. of body weight daily, and it appeared desirable to insure this level of intake during the entire gestation period in order to decrease the hazards of abortion. These values for carotene intakes are in line with those suggested by Boyer *et al.* (170) in 1942, but do not support the figures established by Guilbert and co-workers (171). Baker and co-workers (172) found that 60 μ g. per lb. of body weight was insufficient to maintain liver stores or plasma vitamin A levels of beef cows during six months gestation. The vitamin A in the plasma and liver of the calf was closely associated with the carotene in-

take of the dam during lactation but was also influenced by liver stores of the cow at parturition. Aschaffenburg, *et al.* (173) reported that lecithin was of little help in the absorption of vitamin A in the young growing calf, which is not in agreement with the results obtained in the author's laboratory [Kastelic *et al.* (174); Hansen *et al.* (175)]. Parrish *et al.* (176) studied the properties of colostrum and its absorption and digestion in the young calf. They reported that protein was less than 90 per cent digestible while vitamin A was absorbed to the extent of 81 to 95 per cent during the early period of the calf's life whereas the absorption of carotenoids was only 38 to 65 per cent. The studies of James & Elgindi (177) are of interest in this respect. These investigators found that casein, lactalbumin, gluten, and zein were not equivalent in their effect on carotene absorption and storage in the young rat.

STRESS

Stress continues to be used as a tool to sharpen nutritional research. Its use as a tool of investigation is justified where metabolic nutrients are concerned, but it is of doubtful use as a practical adjunct in nutrition.

Thyroid preparations, thyroprotein, and thyroxin itself have been used in numerous investigations to stimulate milk and egg production. Numerous short term feeding experiments have been conducted with milk cows on the premise that production of milk is correlated with the metabolic rate. These studies indicated that the feeding of the proper level of thyroprotein to milking cows does increase milk and butterfat production with concomitant loss of body weight. Similar findings were reported by Swanson & Hinton (178) who fed cows thyroprotein for 100 days beginning after the peak of milk flow (one lactation). In a long term experiment Thomas & Moore (179) studied the effect of feeding thyroprotein to cows from the 50th day post-partum until 90 days before the next expected parturition. The length of time the thyroprotein was fed ranged from 319 days maximum to 228 days minimum for cows finishing from two to six lactations under treatment. The results of this study differ from those obtained with short term experiments in that the total milk or butterfat production over the entire lactation was no greater than that obtained from untreated cows similarly fed and the rate of production was decreased in subsequent lactations. The initial increased production of milk during the thyroprotein feeding caused a greater weight loss than that seen in comparable, untreated cows; this loss was quickly regained when the thyroprotein supplementation was stopped. There was no extra gain in milk production or fat test unless extra TDN² was fed. The feeding of thyroprotein had no effect on the calving interval, but calf mortality was increased. These results emphasize the necessity for long term experiments in evaluating the practical gain to be obtained from stimulatory additives and caution against their continued long time use.

The hyperthyroid rat continues to provide a useful assay for detecting new growth factors. Tria & Barnabei (180) found activity in an acetone ex-

tract of dried liver which was not cobalamin. The factor was heat stable, dialyzable, and not destroyed by acid or alkali. Cottonseed oil when fed at 10 per cent level was observed by Ershoff (181) to result in better survival and growth than a fat-free or 2 per cent fat diet. Defatted liver residue was as effective as cottonseed oil when both were fed at a 10 per cent level. The addition of B vitamins to the 10 per cent fat diet completely counteracted the growth retardation by the hyperthyroid diet. Bosshardt & Huff (182) fed growing mice a low fat diet (0.5 per cent) which allowed normal growth. Then by adding stress-producing agents such as succinylsulfathiazole, streptomycin, quinacrine hydrochloride (atabrine), desiccated thyroid, or triacetin caused a retardation of the growth rate. The retarded growth could be prevented if the stress-containing ration was supplemented with fat or fatty acids. These workers conclude tentatively that the stresses employed interfere with the synthesis of long-chain fatty acids. Elvehjem and co-workers (183) found that mammalian muscle, liver, yeast, and alfalfa were good to excellent sources of their growth factor. Graham *et al.* (184) noted that beef muscle was the only protein which they studied that gave results similar to defatted liver residue.

The nonselective filtration of elements by the thyroid has been reported earlier. Baumann *et al.* (185) recently reported studies using radioactive isotopes in which it was shown that all of the elements of group VII studied were concentrated in varying degree by the thyroid. The affinity of the thyroid was greater for the heavier elements of the group and the affinity for Tc, I, and Re is of a similar order of magnitude and greater than that for Mn and Br. Chronic iodine deficiency has been implicated by Bielschowsky (186) as a cause of neoplasia in the thyroid and pituitary of aged rats. It is known that dried distillers' solubles, B₁₂, and a whey factor stimulate growth. Manna & Hauge (187) observed that the growth rate of rats fed dried distillers' solubles was significantly greater than that of animals fed orotic acid isolated from spray dried distillers' solubles. From the absorption spectra observations the authors postulate that orotic acid is closely related to vitamin B₁₂ or that orotic acid is a part of the vitamin B₁₂ molecule. Young, Gillis & Norris (188) obtained an alcohol-soluble extract from peanut meal which improved the growth of chicks fed a cornstarch purified casein diet with supplements of methionine, glycine, arginine, vitamins, and essential minerals. Hanson and co-workers (189) found that a forage juice concentrate from alfalfa stimulated growth of chicks fed either a semi-purified diet or a natural diet. Berg & Baylock (190) reported that the addition of 5 per cent fish solubles added to a diet of a soybean protein, sucrose properly balanced, greatly increased the rate of growth of chicks. Peterson, Weiss & Pappenhagen (191) tested various commercial feeds and found that supplements of fish meal, fish solubles, various whey products, and liver preparations resulted in growth increases in chicks of approximately 40 gm. in four weeks. The concentration and partial separation of this fish soluble factor was achieved by Weiss, Pappenhagen & Peterson (192). Miller (193) reported a factor present in

cornstarch which he considered to be a new and unidentified factor for the duckling. The deficiency of this factor manifests itself in the duck by unkempt appearance, poor growth rate, poor feathering, and acute pancreatic fibrosis. Catron *et al.* (194) fed a synthetic milk formula to baby pigs and found that disease-free pigs could not be raised without lard in the diet.

MISCELLANEOUS

Fantastic claims for the use of dietary chlorophyll preparation in the control of body odors have been made based upon subjective measurements dependent upon odor-sensitive observers. Langley & Bolin (195) attempted to develop an osmoscope, a device for mixing gases in which an odoriferous gas was mixed with a control gas to determine the effect upon the degree of odor removal. This method is also dependent upon an odor sensitive panel of observers. The medical literature records evidence that chlorophyll or chlorophyll fractions when placed in intimate contact with injured tissues stimulates the granulation process in healing [Gruskin (196); Lesser (197); Bowers (198)]. The healing action in such diseases as sinusitis, mastoiditis, and other suppurative diseases is reported to be hastened by contact with chlorophyll. Concurrently with the stimulation of the healing process there would be an automatic reduction in suppuration and consequently in the foul odor. The claims for "chlorophyll" as a general biological deodorant rest upon very slim scientific evidence.

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BIOCHEMISTRY OF CANCER¹

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The trend toward an even greater emphasis on cancer research continued during the past year. More than 1,000 publications in this field were reviewed by the author. A majority of these papers are biochemical and could be justly included in a discussion of this nature. However, there are space limitations on this review, and many important studies that could certainly be classified as biochemical must be omitted. For these omissions the author expresses sincere apologies. Papers bearing on the direct application of biochemical methods to clinical cancer as well as most of the literature reports on chemotherapy, tissue culture studies, immunology, and the virus aspects of cancer will not be included in this review. The cancer sections in the forthcoming volumes of the *Annual Review of Biochemistry* (Vol. 24) and of the *Annual Review of Medicine* (Vol. 6) be will prepared by Dr. Alexander Had-dow, who is eminently qualified to consider the chemotherapeutic and medical aspects of the recent developments in the cancer field.

The reader's attention is called to several new publications and abstract services that appeared during 1953 in the cancer field. One of these, *Advances in Cancer Research* (1), is a publication that will be of interest to the biochemist. Gomasasca & Quarti (2) are the authors of a two volume publication on the mechanisms of carcinogenesis and the biology of neoplastic tissues. The first volume of *Leukemia Abstracts* (3) appeared in 1953, and a section on Cancer was added to *Excerpta Medica* (4) during the year.

An attempt has been made in preparing this chapter to review for the reader the role of biochemistry in the overall field of cancer research. It will become evident that considerable progress is being made. As in all major fields of research endeavor, there are certain inconsistencies and conflicting reports which must be considered. It is hoped that the author's selection of papers and the interpretation thereof may provide the reader with an accurate and perhaps somewhat critical evaluation of the progress achieved in cancer research during the past year. The subject will be reviewed under three main divisions: (a) Biochemistry of Carcinogenesis, (b) The Chemistry and Metabolism of Tumors, and (c) The Effect of Tumors on the Composition and Function of Normal Tissues.

BIOCHEMISTRY OF CARCINOGENESIS

Attention is called to a new text concerning the chemical induction of cancer (5). Coman (6) has prepared an excellent review on the mechanism of the origin and distribution of blood-borne tumor metastases. Many of the

¹ The survey of the literature pertaining to this review was completed in November, 1953.

factors related to the origin of bone tumors have been reviewed by Johnson (7). Further studies have been reported by Pullman *et al.* (8, 9) on the electronic structure necessary for carcinogenic activity of the aromatic hydrocarbons. Energies of polarization for the K and also the L regions of a number of compounds were calculated. For a compound to be carcinogenic, the K region must be very active and the L region, if present, must not be too active, according to the authors. This generally appears to be the case with the limited number of hydrocarbons tested. They state that the carcinogenic activity of 1,2-benzpyrene which has no L region and a weak K region may be explained by steric factors. If should be pointed out, however, that this structure is feebly active as a carcinogenic agent. Several other reports have also appeared regarding the mechanism of action of carcinogenic substances (10 to 13). Druckrey & Schmahl (14) reported the formation of sarcomas in 13 of 50 rats that had hydrocellulose implanted under the skin. This carcinogenic activity was attributed to a bonding of cell proteins on the active surface of the hydrocellulose. Implanted caprolactum discs, another surface active macromolecule, also produced sarcomas in rats. Oppenheimer *et al.* (15) have also obtained a high incidence of tumors by imbedding a variety of plastics in the tissues of mice and rats. A commercial cellophane, exhaustively extracted with methyl alcohol, was the most effective in producing tumors. Hofbauer (16) advanced the concept that a series of biochemical changes conditions the uterine mucosa to the development of carcinoma. Alterations in the pituitary-adrenal-gonad relationship are involved in this concept. Electrocardiac potentials are not pathognomonic of female genital-tract carcinoma, according to Max *et al.* (17), as previously reported by other investigators. Nordling (18) has theorized that cancerous cells contain not one but a number of mutated genes. The accumulation of mutations may be expected to increase with age and with cell proliferation.

COCARCINOGENESIS

In contrast to earlier theories of cocarcinogenesis, Shubik & Ritchie (19) have observed that increases in the number of applications of hydrocarbons followed by croton oil did not give rise to increasing numbers of tumors. Either one, two, or three skin applications of 9,10-dimethyl-1,2-benzanthracene, followed repeatedly by croton oil, in C3H mice did not result in any differences in the tumor incidence. In Swiss female mice decreased numbers of tumors were recorded with increasing numbers of the hydrocarbon applications. These findings are difficult to explain by the cocarcinogenic concept. It is improbable that croton oil following several applications of the hydrocarbon counteracted the initiating action of the carcinogen. More likely second and third applications of the hydrocarbon altered the tissue so that they became refractory to the croton oil. Klein (20, 21) painted DBA mice with methylcholanthrene, and, two weeks later, croton oil was applied to the same site. One to twenty paintings with the croton oil did not stimulate tumor formation; however, a cocarcinogenic effect was observed with 30

applications. It was also noted that croton oil was more effective as a promoter when applied continuously rather than intermittently. Cortisone, which inhibits mitotic activity in normal mouse skin but not in hyperplastic epidermis resulting from hydrocarbon treatment, had no effect on the hyperplasia resulting from the repeated applications of croton oil to the skin of the mouse (22). Further, the hyperplasia produced by applying croton oil to skin previously painted with a carcinogenic hydrocarbon reacted in the same manner to cortisone. These findings do not support the observations of Salaman & Gwynn (23) that a different hyperplasia is produced when croton oil is applied to skin previously treated with carcinogenic hydrocarbons. Iverson *et al.* (24) have carried out further studies on the effect of croton oil on skin previously exposed to carcinogenic agents.

The recent reports on cocarcinogenesis, while significant, do not extend our knowledge of the basic mechanism involved. Dose levels of the cocarcinogenic agents, the timing and frequency of croton oil painting, and the species are factors that must be given more consideration in future studies. Another clinical case of possible cocarcinogenic origin was reported (25). A petroleum corporation employee developed an epidermoid carcinoma at the site of trauma. Prolonged occupational exposure to oil is suggested as the initiating agent with trauma and healing as the promoting agent.

CARCINOGENIC AGENTS

Azo compounds.—A review of the chemical constitution and carcinogenic activity of the azo compounds has been written by Badger & Lewis (26). Four of five dogs fed 5 mg./kg./day of *o*-aminoazotoluene for 30 to 62 months developed tumors. Two of the animals had tumors in the urinary bladder and two had tumors in the gall bladder and liver. These same investigators noted that dogs fed 20 mg./kg./day of 4-dimethylaminoazobenzene (4-DAB) developed bladder tumors after approximately 40 months. The 4-DAB² at this high level was quite toxic. Lower levels of feeding (5 mg./kg./day) for 63 months did not produce tumors [Nelson & Woodard (27)]. These findings would suggest that the azo compounds are not so species specific as is generally believed. Perhaps the extended administration of larger doses may induce tumors in tissues and species now thought to be resistant to these azo compounds. Bertrand *et al.* (28) have made a histological study of the tumors produced in the rat with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-4-DAB). They found a variety of hepatic tumors as well as splenic tumors. Whether or not the splenic tumors were primary or the result of metastases from the hepatic tumors cannot be ascertained. In investigations involving guinea pigs, Haranghy *et al.* (29) found that vari-

² The following abbreviations are used: 2-AAF for 2-acetylaminofluorene; ACTH for adrenocorticotrophin; 4-DAB for 4-dimethylaminoazobenzene; 3'-Me-4-DAB for 3'-methyl-4-dimethylaminoazobenzene; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; RNA for ribonucleic acid; TPN for triphosphopyridine nucleotide.

ous combinations of cholesterol, 4-DAB (diet), and benzpyrene resulted in a polymorphocellular sarcoma in one animal. Other guinea pigs developed fibrosarcomas after treatment with melanin, cholesterol, and benzpyrene. The findings of these investigators would be significant if they had definite evidence that either cholesterol or 4-DAB accentuated the action of benzpyrene. It is difficult to determine the actual or the contributory factors involved in the induction of tumors in these complicated studies. Young rats given relatively large quantities of 4-DAB, i.e., 200 to 500 mg. over a period of two weeks apparently metabolized the dye within two weeks and then grew normally, according to Druckrey (30). After latent periods, up to 800 days, the rats developed tumors. The author points out that tumors appearing in the aged may have had their origin in youth. Von Euler *et al.* (31) have reviewed studies involving 4-DAB and report some preliminary observations regarding the origin of hepatomas.

Miller and co-workers (32) synthesized 2-fluoro-, 2',4'-difluoro-, 2',5'-difluoro-, 3',5'-difluoro-, and 2',4',6'-trifluoro-4-DAB. All of these compounds were found to be more carcinogenic than the parent dye in the rat. From these findings the authors state that no position on the prime ring of 4-DAB is directly concerned in the carcinogenic process. This conclusion is also supported by observations of these investigators on protein-bound dye. The introduction of an ethyl group into the azo molecule with one exception decreased the carcinogenic activity of the parent compound. The activity of 4'-ethyl-N-methyl-*p*-aminoazobenzene was as great as that of N-methyl-4-aminoazobenzene. Other ethyl substitution in the prime ring or on the amino group of 4-DAB decreased or abolished carcinogenic activity [Sugiura, Kensler & Crossley (33)]. Marshall (34), by prolonged vital staining with the diazo dyes, trypan blue, or Evan's blue, produced malignant tumors of the reticular tissue of the rat. The author points out that there may be danger of carcinogenic action when Evan's blue is used to determine blood volumes in man. Simpson (35) observed proliferation in the portal tracts and also reticulum cell carcinoma of the liver in rats injected for prolonged periods with trypan blue. Mice painted with 4-dimethylaminoazobenzene-1-azo-2-naphthalene for approximately 240 days developed a high incidence of hepatomas and hemangioendotheliomas and some cholangiomas [Mulay & Saxen (36)].

Acetylaminofluorene.—In connection with the observation of Miller *et al.* (32) that fluoro substitution in the 4-DAB molecule enhances carcinogenic activity, it is of interest to point out that 7-fluoro-2-acetylaminofluorene is a stronger hepatocarcinogen than the parent compound (37). Morris & Eyestone (38) were able to induce tumors of both the liver and bladder in four of five dogs given 2-acetylaminofluorene (2-AAF) orally for 68 to 91 months. These interesting observations from this study are in complete agreement with those reported by Nelson & Woodard (27) involving tumor formation in dogs fed *o*-aminoazotoluene. These combined findings, along with those of Allison *et al.* (39), suggest that the dog is probably susceptible

to a variety of chemical carcinogens. A most interesting study has been reported by Mosonyi & Korpassy (40). These investigators found that tannic acid greatly enhanced the hepatocarcinogenic action of 2-AAF. The simultaneous administration of tannic acid accelerated liver cirrhosis, which was considered to be an important factor in liver carcinogenesis.

Tuba *et al.* (41) reported that female rats fed Purina diets containing 4-acetylamino-biphenyl did not develop tumors in the mammary glands; however, hyperplastic nodules did eventually appear. Sandin and co-workers (42), on the other hand, did observe tumors in rats when this agent was fed in a grain diet. It would appear of interest to determine what factors were present in the Purina diet that inhibited the carcinogenic action of this compound or the stimulating factors present in the grain diet.

Hydrocarbons and related compounds.—The composition and action of a group of aromatic carcinogenic compounds have been studied by Druckrey *et al.* (43). Sarcoma appeared in guinea pigs given a single injection of 9,10-dimethyl-1,2-benzanthracene [Mosinger (44)]. Heston & Deringer (45) have reported the induction of pulmonary tumors in guinea pigs by the intravenous injection of dibenzanthracene. These investigators determined the occurrence of pulmonary tumors in several strains of mice following the intravenous injection of dibenzanthracene (46). Methylcholanthrene, applied locally to the skin, produced a fibroma in the feather follicle of the duck [Rigdon (47)]. This agent also, when injected into the wall of the stomach, induced tumors of the glandular stomach in several strains of mice tested (48). DBA mice were refractory to the induction of all types of malignant tumors by this technique. Andervont & Dunn (49) administered methylcholanthrene orally to strain DBA_{1/2} mice without the mammary tumor agent. Mammary gland tumors were induced in the females, and leukemias appeared in both sexes.

Further studies have appeared regarding the carcinogenic activity of metabolic products of benzpyrene (50, 51) and of 2-amino-1-naphthol and amino-2-naphthylamine (52). The addition of 3,3'-dihydroxybenzidine to the diet of rats caused the development of hepatomas, adenocarcinoma of the colon, carcinoma of the sebaceous glands, squamous cell carcinoma of the stomach, and neoplastic changes in the bladder [Baker (53)]. *p*-Benzoquinone applied to the skin of mice, produced papillomas but no carcinomas [Tiedemann (54)]. Buu-Hoï *et al.* (55, 56) have made further studies of the carcinogenic action of nitrogenous bases related to the hydrocarbons. Neither 10-methylbenz(a)acridine nor tetraphenylmethane was carcinogenic when administered to rats.

Nitrogen mustards and irradiation.—Tumors of the pituitary gland have been induced by exposing mice to ionizing radiations (57, 58). Heston and associates (59) observed significant increases in the occurrence of pulmonary tumors in strains of mice injected with nitrogen mustards. Mice that received 900 r x-radiation with their spleens shielded, and subsequently were injected with nitrogen mustards, had the same pulmonary tumor incidence as the

controls and the mice that received irradiation alone, but fewer tumors than the mice that received nitrogen mustards alone. There was no additive effect of radiation and nitrogen mustards in the induction of pulmonary tumors in these mice as would be predicted on the basis of the somatic mutation hypothesis. X-radiation appeared to inhibit the carcinogenic action of the nitrogen mustards. It is of interest to note that nitrogen mustards have been shown to partially inhibit the hepatocarcinogenic activity of 3'Me-4-DAB (60). Richardson *et al.* (61) and Meehan *et al.* (62) have observed that the carcinogenic activity of this same azo compound could be inhibited by the administration of methylcholanthrene. This inhibitory effect observed with certain combinations of carcinogens may be mediated through the pituitary-adrenal system. X-radiation, nitrogen mustards, and methylcholanthrene produce alterations in the histopathological and chemical constitution of the adrenal cortex. This effect of pituitary-adrenal function in mediating carcinogenesis will be considered in more detail in a subsequent section of this review.

Heston (63, 64) has continued studies on the carcinogenic action of both nitrogen mustards and sulfur mustards. Either agent, injected subcutaneously in various strains of mice, induced neoplasms at the site of administration as well as in distal tissues. C3H mice, injected with nitrogen mustards, exhibited a significant increase in the occurrence of pulmonary tumors. Rats, protected against lethal doses of x-radiation either by parabiosis or *p*-aminopropiophenone, developed malignant tumors within approximately six months following radiation (65, 66). No tumors appeared in the nonirradiated partners. It is apparent that both ionizing radiations and the nitrogen and sulfur mustards are potent carcinogenic agents. While the two agents are similar in many respects their action on living systems are apparently not additive in the induction of neoplasms.

Hormones.—Rawson (67) and Sampey (68) have reviewed the role of the estrogens in producing tumors of the breast, cervix, uterus, testes, and lymph nodes. These reviews also consider tumors of the ovaries, adrenals, breast, thyroid, etc., resulting from the administration of pituitary hormones.

Mice injected daily with desoxycorticosterone acetate for six months developed fibrosarcoma and myosarcoma [Mirand *et al.* (69)]. These findings are of interest since most other investigators have reported that administration of this corticoid had no tumorigenic effect. Tumors were induced in the kidneys of intact and gonadectomized male hamsters by prolonged treatment with diethylstilbestrol [Kirkman & Bacon (70, 71)]. Subcutaneously implanted pellets of stilbestrol or estradiol induced tumors when given as above. Ethinyl estradiol and 7-methyl-bis-dehydrodoisynolic acid were ineffective. Dunning, Curtis & Segaloff (72) conducted a thorough study on the effects of estrone pellet implantation in four different inbred strains of rats. Mammary cancers developed in three of the four inbred strains treated with estrone; however, the incidence was somewhat lower

than that observed for rats of the same strains treated with a similar dose of diethylstilbestrol. In the estrone treated series, bladder carcinoma was observed more frequently in the strain (Copenhagen) that proved resistant to the mammary cancer. One of the strains (August) that developed a high incidence of mammary cancer also had a relatively high percentage of adrenal tumors. Mammary tumors occurred in 12 of 60 agent-free C3H₁ male mice implanted with pellets of diethylstilbestrol in cholesterol [Heston *et al.* (73)]. Hepatomas also appeared in 14 of the mice approximately 20 months after the implantation of the estrogen.

Iglesias *et al.* (74) point out that tumors in intrasplenic ovarian grafts in castrate animals presumably are the result of excess pituitary gonadotrophic activity. This occurs since the ovarian steroid hormones that normally control the output of the pituitary gonadotrophins are inactivated in the liver before reaching the general circulation. In the current studies, intrasplenic ovarian grafts were made in castrate guinea pigs, and minute quantities of estrogen were administered over a 26 month period. Ovarian tumors were produced in the grafts when estrogen was absent from the general circulation, and the ovarian tumors also appeared when estrogens were present in sufficiently high concentrations to exert at least partial control over the pituitary. It was concluded that ovarian tumorigenesis could occur without complete suppression of estrogenic activity in the body. A tumor incidence of 81 per cent was observed when ovaries from young BALB/c mice were transplanted into BALB/c X DBA F₁ hybrid castrates. An almost identical tumor incidence was observed when ovaries from young BALB/c mice were transplanted into young castrates of the same strain [Klein (75)]. Biskind *et al.* (76) have demonstrated that administration of gonadotrophin accelerates the development of both luteoma and granulosa cell tumors when an ovary is transplanted into the spleen of a castrated rat.

Other carcinogenic agents.—The results of further studies on the induction of pulmonary tumors by urethane have been reported (77, 78). Moore *et al.* (79) produced pituitary tumors in mice by the addition of propylthiouracil to the diet. All of the mice also developed carcinomas of the thyroid gland. Thyroid tumors resulted from the administration of radioactive iodine alone or in combination with methylthiouracil, according to Doniach (80). This investigator stated that the doses of iodine¹³¹ used in the treatment of Graves' disease may eventually prove carcinogenic. Administration of either zinc or cobalt to rabbits previously fed a diet of oats and bran resulted in the formation of lymphosarcoma in the lumbar adipose tissue [Thomas & Thiery (81)]. Dietary factors, along with the metabolism of lipids, appear to be involved in this carcinogenic process. Carleton *et al.* (82) produced testicular teratomas by injecting zinc chloride solution into the testes of young roosters. Schoental (83) has confirmed the carcinogenic action of the Senecio alkaloids. Rats subjected to intermittent feeding with these alkaloids developed extensive liver changes which led to liver tumors.

Hepatic tumors appeared in rats fed diets containing ethionine (0.2 to 0.5

per cent) for 50 to 100 days. The tumors were of both hepatic and bile duct origin; however, the malignancy was not ascertained [Popper *et al.* (84)]. It will be of interest to determine whether the ethionine produced this effect directly, or indirectly, as an amino acid antagonist. Levy *et al.* (85) have investigated the effect of ethionine on tumor growth and on the concentration of free amino acids in the liver.

Tumors in plants and insects.—Severance of the recurrent nerve in the insect *Leucophaea Maderae* resulted in a high incidence of tumors in the stomach and in the salivary glands [Scharrer (86)]. Plaine & Glass (87) found that the incidence of melanotic tumors in *Drosophila* increases linearly with increasing oxygen concentration at the time of irradiation. The incidence of these tumors was also increased slightly by exposure of the embryos for 10 min. to pure oxygen without x-ray treatment. 1,2,3,4-Diepoxybutane produced mutations when injected into the hemocoeli of *Drosophila*. Melanotic neoplasms also occurred in progeny of the treated flies, thus providing some further evidence for the mutation hypothesis of the origin of cancer [Bird & Fahmy (88)]. Klein *et al.* (89) have investigated the role of crown-gall nucleic acids in the transmission of crown-gall tumors. Studies were also carried out on the desoxyribonucleic acid and ribonucleic acid concentrations of stem tissues of the broad bean at various times during the development of bacterial tumors and auxin-induced proliferation (90). Klein & Link (91) have observed that auxin is a promoting agent in the transformation of normal cells to crown-gall tumor cells.

Lung carcinogenesis.—Most authorities now agree that there is an increase in the incidence of lung cancer (92, 93). There is somewhat less agreement as to the factors responsible for this increase. Occupational factors are undoubtedly involved to some extent (92, 94). Boemke (95) has discussed the formation of lung carcinoma from asbestos inhalation. Several investigators have reported a statistical association between smoking and carcinoma of the lung (92, 96, 97). Other reports of this type have appeared during the last five year period. Wynder *et al.* (98) in simulated smoking of cigarettes, condensed the smoke in flasks immersed in dry ice ethanol. Solutions of the condensate were painted on the backs of CAF mice. Half of these mice developed papillomas and approximately 16 per cent developed carcinoma at the site of tar administration. These findings, while suggestive that cigarettes do contain or produce carcinogenic substances upon combustion, cannot be related directly to the formation of lung tumors in man. Using strain A mice, Essenberg (99) found that exposure to cigarette smoke (one cigarette every hour for 12 hr./day) for 14 months resulted in a lung tumor incidence of 91 per cent. Control mice had an incidence of 59 per cent. Several years ago Lorenz *et al.* (100) exposed strain A mice to tobacco smoke for 28 to 250 days. They obtained the same lung tumor incidence in both the control and smoke-treated mice. Weanling white rats were exposed by Haag *et al.* (101) to cigarette smoke every half hour, 14 times daily, for the life span of the animals. These investigators did not observe any lesions in these

animals. Since the above experiments were not carried out under identical conditions, no further explanations can be offered for the apparent discrepancies reported. It should be pointed out that Essenberg used strain A mice, a strain that has a high spontaneous incidence of lung cancer. Exposure of this strain of mice to other agents such as the nitrogen mustards and ionizing radiations also results in an increased lung tumor incidence. It would be of interest to determine the effect of other kinds of smoke on the incidence of lung cancer in strain A mice. Until further studies have been carried out with other mouse strains and with other species it is difficult to state that cigarette smoke is a carcinogenic agent. From Essenberg's results (99), which have not been confirmed by other investigators, we can only conclude that cigarette smoke promotes the development of lung cancer in a strain of mice already highly susceptible to the disease. What the effect of cigarette smoke would be on other animals without this inherent disposition towards lung cancer remains to be discovered.

The increasing incidence of lung cancer in man remains a challenging problem to those in cancer research. It would appear unwise to place the entire blame on cigarette smoking on the basis of the evidence now at hand. Careful studies in search of other factors associated with our environment should be conducted. Certainly, extensive and long term investigations should also be carried out to determine the effect of smoking and of smoke or tobacco by-products on the tissues, organs, and physiological and biochemical functions in many animal species.

METABOLISM OF CARCINOGENIC SUBSTANCES

Mueller & Miller (102) studied the demethylation of 3-methyl-4-monomethylaminoazobenzene by rat liver homogenates. Oxygen, TPN,² DPN,² and hexosephosphate are required for optimal activity. The apparent pathway for this demethylation probably involves the formation of an intermediate N-hydroxymethyl derivative. The investigators could account stoichiometrically for the metabolized dye as primary aminoazodye and formaldehyde. The postulated N-hydroxymethyl intermediate may be the form of the dye that combines with intracellular proteins. The synthesis of 4-DAB, labeled with C¹⁴ in either benzene ring, has been carried out by MacDonald, Miller & Miller (103). Preliminary findings on the metabolism of these radioactive compounds in the rats were also reported. Most of the C¹⁴ was excreted in the urine and feces with only a small percentage appearing in the respired air. These workers have also synthesized 4-DAB, 4-monomethylaminoazobenzene, and their 3-methyl and 4'-methyl derivatives, all labeled with C¹⁴ in the N-methyl position (104). When these labeled azo compounds were administered to rats, 50 to 70 per cent of the C¹⁴ was expired as C¹⁴O₂ within 48 hr. Ten to thirty per cent was excreted in the urine and 4 to 9 per cent in the feces. The specific activities of protein, protein-bound serine, and choline, isolated from the liver, were the same regardless of the dye administered. The metabolism of the N-methyl groups of strong and weak azo

carcinogens is apparently the same. The methyl groups are probably oxidized to formaldehyde. Von Euler *et al.* (105, 106, 107) have carried out further studies on the metabolism of 4-DAB. The reactions of pyruvic acid and other important metabolites with *p*-phenyldiamine, *o*-, *m*-, and *p*-amino benzoic acids have also been investigated. 4-DAB is solubilized to a considerable extent in serum, according to Wunderly (108). Relatively large quantities of the dye are bound and transported by the serum proteins as indicated by filter paper electrophoresis studies. Studies on carcinogenesis employing the serum solution of 4-DAB are reportedly in progress.

Additional information on the metabolism of 2-AAF² has been obtained by Dyer *et al.* (109). 2-AAF-N¹⁴ was administered to rats and a close similarity between the distribution and excretion of this compound and the earlier observations (110) with 2-AAF-9-C¹⁴ was noted. Following the Miller technique, Dyer *et al.* (109) found that the N¹⁴ of 2-AAF was bound to the protein fractions of the liver of rats. A quantitative spectrophotometric method for determination of 7-hydroxy-2-acetylaminofluorene has been reported by Damron & Dyer (111). Gutmann & Peters (112) have carried out metabolic studies of 2-benzoylamino fluorene-9-C¹⁴ and 2-AAF-9-C¹⁴ in the rat.

The metabolism of naphthalene and other polycyclic compounds both in intact animals and in rat liver slices has been investigated by Boyland & Wiltshire (113, 114). Heidelberger *et al.* (115) have continued their studies on the metabolism of C¹⁴-labeled dibenzanthracene. Further observations on the intracellular distribution of 3-4-benzopyrene have been published by Calcutt & Payne (116). The distribution of benzidine has been determined following a single intraperitoneal injection in the rat [Baker & Deighton (117)]. Beale & Roe (118) have made ultraviolet absorption studies on *trans* and *cis*-stilbenes.

INTERACTION OF CARCINOGENS OR THEIR METABOLITES WITH TISSUE COMPONENTS

An extensive study of the interaction of dibenzanthracene-9,10-C¹⁴ with tissue components has been conducted by Heidelberger *et al.* (119 to 122). An irreversible binding of the labeled compound or its metabolites to the proteins of a skin fraction containing epidermis and dermis was noted. Binding of the carcinogen to the proteins and other constituents of the submaxillary glands of mice was also demonstrated. The binding occurred with nucleoprotein but not with nucleic acids. Further studies are required before the full significance of these hydrocarbon-protein complexes in the process of cancer induction is understood. This approach, while obviously complicated because of the many fractionations, isolations, and characterizations involved, is worthy of the time expended. From these and related studies with other carcinogenic compounds will develop a knowledge of the essential biochemical combinations which initiate the process of cancer induction.

An isolation and purification of the azo dyes liberated by hydrolysis of liver proteins from rats fed 3'Me-4-DAB has been reported by Brown *et al.*

(123). These investigators obtained evidence to indicate that the polar dye may be bound to the protein through the amino nitrogen of the dye. In a preliminary report, Nye & Luck (124) have presented evidence that would suggest that the carcinogenic azo compounds become attached to the protein by an amino group adding to the benzene ring in the 2 position. The polar dye, obtained from hydrolysates of liver protein, on methylation yielded a derivative with spectral characteristics similar to that obtained from chrysoidin (2,4-diaminoazobenzene) when the latter was similarly treated. The two groups of investigators agree as to the benzene ring of the azo dye that is involved in the protein binding (32, 123, 124). However, final proof for the detailed nature of this binding must be ascertained. Considerable emphasis has been placed upon the binding of azo dyes by the liver proteins as an essential step in liver carcinogenesis. Further studies will certainly be required to establish that the formation of the protein-azo compound complex represents an essential step in this carcinogenic process.

Wunderly has investigated the solubility of 4-DAB (108) and also of carcinogenic hydrocarbons (125) in blood serum. The carcinogenic agents were soaked in filter paper. Serum was subjected to electrophoresis on the paper, and it was observed that the carcinogenic agents were associated with the serum protein components, when isolated by this technique. Burkhard (126) studied the binding of azo dyes by the serum proteins in order to obtain further data as to the possible relation between carcinogenic activity and protein complexing activity. 3'-Me-4-DAB was bound to the greatest extent, closely followed by 4'-methyl-4-aminoazobenzene and 4-aminoazobenzene, indicating little if any correlation with the known carcinogenic activity of these compounds. Dyer *et al.* (109), following the methods of Miller & Miller, have found that the N¹⁸ of 2-AAF-N¹⁸ was bound to a protein fraction of the liver of rats and to a lesser degree to the protein fractions of several other rat tissues.

FACTORS THAT AFFECT CARCINOGENESIS

Endocrine and hormonal factors.—The nutritional and hormonal interrelationships in the development of experimental cancer have been reviewed by Morris (127). The simultaneous administration of either estradiol or diethylstilbesterol with the intravenous injection of 9,10-dimethyl-1,2-benzanthracene to female rats caused an earlier appearance of tumors and also increased the number of tumors [Geyer *et al.* (128)]. Injections of methylcholanthrene into the prostate of castrate rats produced a high percentage of squamous cell carcinoma. A considerably lower incidence was noted when the carcinogen was injected into either intact or testosterone-treated rats [Allen (129)].

Certain of the hormones of the adrenal cortex have been shown to alter chemical carcinogenesis. Schober (130) observed that administration of benzyropyrene to mice injected daily with cortisone developed fewer tumors than the hydrocarbon treated controls. Adrenalectomy or administration of desoxycorticosterone acetate enhanced the tumorigenesis. Boutwell & Rusch

(131), in contrast to the above results, noted that administration of cortisone, in the diet or locally, did not alter the incidence of benzpyrene-induced carcinomas in mice. However, both oral and local treatment with cortisone reduced the incidence of papillomas resulting from the benzpyrene. The effect of cortisone on skin carcinogenesis in mice has also been investigated by Engelbreth-Holm & Asboe-Hansen (132). According to Sulzberger *et al.* (133), the parenteral administration of cortisone increased the incidence of epidermal tumors in mice given methylcholanthrene. The above investigators are not in complete agreement as to the effect of cortisone on carcinogenesis and further studies are required to ascertain the role of this hormone in the process of cancer induction. Several workers are in agreement that cortisone does enhance the success of homologous transplantation of tumors (134, 135). Hoch-Ligeti & Hsu (136) observed that a variety of human neoplasms survived for 20 days or longer in cortisone-treated rats. This decreased resistance of the animal to heterotransplantation was postulated to be attributable to the disappearance of leukocytes that accompany the administration of cortisone.

The formation of postcastration adrenal cortical nodular hyperplasia in both sexes of C3H mice was prevented by hypophysectomy [Ferguson & Visscher (137)]. Griffin *et al.* (138) found that hypophysectomized rats fed diets containing 3'Me-4-DAB did not develop liver tumors or the fatty infiltration, liver enlargement, cirrhosis, etc. that usually precedes the tumor. Adrenal histological changes that accompany liver tumor inhibition in the hypophysectomized dye-fed rats have been observed by Richardson *et al.* (139). Administration of adrenocorticotrophin preparations to the hypophysectomized dye-fed rats resulted in a partial restoration of the carcinogenic process. Cirrhosis and liver tumors were present after 20 weeks of adrenocorticotrophin (ACTH) administration in the dye-fed hypophysectomized rats [Robertson *et al.* (140)]. Testosterone and several adrenal corticoids were without effect in the above respect. Moon *et al.* (141) had previously reported that administration of methylcholanthrene to hypophysectomized rats of the Long-Evans strain resulted in a great reduction in the tumor incidence. It would appear that the pituitary is involved in the process of chemical carcinogenesis. Recent studies carried out in the writer's laboratory have revealed the formation of tumors at the site of either benzpyrene or methylcholanthrene administration in approximately 20 to 30 per cent of hypophysectomized rats of the Sprague-Dawley strain. Also of interest was the observation that administration of ACTH to the hypophysectomized rats injected with either benzpyrene or methylcholanthrene resulted in a considerably higher tumor incidence. While it would appear that pituitary function is involved in certain types of cancer induction there are many other tumors that may be induced in the absence of the pituitary.

Silberberg *et al.* (142, 143) have made observations on the effect of the adrenals and also of the anterior pituitary in carcinogenesis. Castrate mice of two hybrid stocks maintained on diets containing diethylstilbestrol devel-

oped a far greater incidence of breast cancer than the intact animals similarly treated [Huseby (144)]. At autopsy most of the castrate mice were found to have pituitary nodules. These altered pituitaries were associated with lobuloalveolar development in the mammary glands. Sellers *et al.* (145) determined the effect of iodide and thyroid on the induction of adrenal and pituitary tumors by propylthiouracil. Adenomas of the thyroid occurred in all experimental groups and in some instances metastasized to the lung. Pituitaries of rats receiving propylthiouracil and thyroid were greatly enlarged and were frequently adenomatous.

Carcinogenic compounds and other factors.—There is additional evidence that the action of carcinogenic compounds may be altered by the administration of other carcinogenic agents (146). Miyaji *et al.* (147) found that the carcinogenic action of 2-AAF in rats could be inhibited by the simultaneous administration of methyl cholanthrene or several other hydrocarbons. Further studies on the inhibition of 3'-Me-4-DAB by methylcholanthrene (61) have been carried out by Meehan *et al.* (62). Administration of the hydrocarbon before the tenth week of dye feeding was effective in blocking hepatic tumor formation. After the tenth week the methylcholanthrene was ineffective.

Shielding of the spleen prevented the induction of tumors in C57 BL mice exposed to irradiation [Lorenz *et al.* (148)]. Kaplan *et al.* (149, 150) have found that intravenous injection of bone marrow cells inhibited the induction of thymus lymphomas in C57 BL mice receiving x-radiation. The bone marrow presumably favors the recovery of the radiation-damaged thymus.

Nutrition.—Several reviews have recently appeared concerning the possibility of cancer induction by diet alone and also on the role of the diet in modifying the action of other carcinogenic processes (127, 151 to 156). Bielschowsky (157) has observed basophil adenomas of the pituitary and adenomas of the thyroid in old Wistar and hooded rats maintained on low-iodine diets. Rats maintained on iodine-deficient diets for prolonged periods by Axelrod & Leblond (158) also developed a high percentage of thyroid neoplasms. In iodine deficiencies, excessive amounts of thyrotrophic hormone are released from the pituitary, thus stimulating the thyroid gland. Prolonged stimulation of this type may be the cause of the thyroid neoplasms.

Tannenbaum & Silverstone have reported on several phases of their study of dietary factors that affect the genesis and growth of tumors. Levels of 2, 4, or 8 per cent standard salt mix did not alter the incidence, mean time of appearance, rate of spontaneous mammary tumor, growth, or carcinogen-induced skin tumors in the mouse (159). In another study, underfeeding or caloric restriction (decrease of carbohydrate) of mice with mammary carcinoma increased their life span, decreased the rate of growth of the tumors, and decreased the frequency of lung metastases. These restricted animals also had lower body weights than their unrestricted controls (160). The genesis of spontaneous mammary carcinoma in C3H mice was hindered by

decreases in the dietary protein below certain critical levels (161). Spontaneous hepatomas form faster in C3H mice fed diets containing 18 per cent casein than on diets containing 9 per cent casein. The addition of sulfur amino acids to diets containing the lower protein level brings the hepatoma development up to that observed in the higher casein level. These investigators found that the genesis of the hepatomas was dependent on the proportion of balanced protein and not upon the specific action of sulfur amino acids in the diet as would be indicated by the above casein studies (162). Waxler *et al.* (163) have found that C3H mice made obese with gold thio-glucose show an earlier appearance of spontaneous mammary tumors than was observed in control mice of normal weights. The obese mice also consumed more food than the controls.

A recent review (164) has appeared on the relation of kwashiorkor to nutritional cirrhosis and liver carcinoma. Koyanagi (165) has also reviewed the relation of riboflavin to liver cancer. Certain *Neurospora* mutants appear to utilize azo dyes in place of riboflavin, according to results obtained by Salzberg (166). Considerable mycelial growth was observed in riboflavin-free media which contained either 3'-Me-4-DAB or the more soluble 3'-methyl-4-monomethylaminoazobenzene. These findings suggest that certain metabolic interrelations between riboflavin and the azo compounds may explain the role of the former in azo dye carcinogenesis. Biotin and also oxy-biotin failed to exert a procarcinogenic effect in liver tumor formation by 4-DAB [Axelrod & Hofmann (167)]. Several previous workers have reported that biotin does enhance azo dye carcinogenesis.

The effect of high and low protein diets on the carcinogenic action of aminostilbene derivatives has been published by Elson (168). Low protein intakes favored the development of cholangiomas but not of other tumors in rats treated with 4-dimethylaminostilbene. The dietary protein effect was not observed in rats that were given 2'-chloro-4-dimethylaminostilbene. Engel & Copeland (169) have also discovered that low-protein diets enhanced the carcinogenic activity of 2-AAF. Weanling rats fed on low-casein diets containing 2-AAF had an 86 per cent liver tumor incidence as compared to only 12 per cent incidence when the casein level was increased two or three fold. Mice fed on a 50/50 bentonite-semisynthetic diet failed to grow and developed fatty livers [Wilson (170)]. The addition of choline or methionine partially restored growth and prevented fatty liver formation. The bentonite, as a cation-exchange silicate, apparently removed choline from the intestinal contents. The continued feeding of the bentonite-basal diet resulted in the development of a high incidence of hepatomas (171).

EFFECTS OF CARCINOGENIC AGENTS ON THE COMPOSITION AND FUNCTION OF NORMAL TISSUES

The localization of enzyme systems within mitochondria has focused more attention on the role of this particular fraction in carcinogenesis. Hogeboom *et al.* (172) have ably reviewed the biochemical properties of the

mitochondrial as well as of other intracellular components of liver. Striebach and associates (173) have observed a 150 per cent increase in the numbers of mitochondria in the livers of rats fed the noncarcinogenic dye, 2-methyl-4-dimethylaminoazobenzene. Feeding 3'-Me-4-DAB resulted in a 41 per cent decrease in the number of liver mitochondria. Livers of rats fed the basal diet had an average of 228×10^6 nuclei per gram while livers of rats fed 3'-Me-4-DAB had an average of 500×10^6 nuclei per gram. Further analysis of the results obtained in this study indicated that there was a decrease in the cytoplasm volume rather than nuclear change in the livers of rats fed the 3'-Me-4-DAB. The average liver of rats fed this dye contained 55 per cent parenchymal tissue, 16 per cent bile-duct tissues, 29 per cent unclassified tissue. The values for the same tissues from livers of rats fed the basal diet were 90.0, 0.04, and 9.6 per cent respectively. Livers of rats fed 3'-Me-4-DAB had lowered activities with respect to succinoxidase, DPN-cytochrome-*c* reductase, octanoic-oxidase, and uricase, which are all present in mitochondria [Schneider *et al.* (174)]. This decrease in enzyme activities was associated with the decrease in mitochondrial population that occurs with the administration of this dye. Increases in the activities of these enzymes were noted in the livers of rats fed 2-methyl-4-dimethylaminoazobenzene. The authors conclude that the mitochondria are involved in the carcinogenic process in liver but that the role of the mitochondria in this process is more complex than earlier data indicated. The reader's attention is called to new methods for counting mitochondria in tissue homogenates (175, 176) and to a method described by Novikoff *et al.* (177) for the separation of rat liver mitochondria of different size and biochemical properties.

Studies on the intracellular composition of livers from rats fed 2-AAF have been reported by Laird & Miller (178). Values for protein, nucleic acids, and riboflavin, in homogenates, nuclei, large and small granules, and in supernatant fluid fractions, after 4, 7, 14, 25, and 27 weeks are given. Laird (179) has also investigated nuclear changes occurring in the livers of rats treated with thioacetamide. Administration of the drug did not alter the DNA² content of the average nucleus; however, it did result in increases in both protein and RNA² in the nucleus. According to Thompson *et al.* (180) the DNA phosphorus per nucleus was significantly decreased in the livers of rats following prolonged feeding of 4-DAB. This conclusion was based on a rather small number of rats fed the dye for five months; some rats had liver tumors and some had not. These investigators also reported that the carcinogen caused an increase in the total number of cells in the liver of the order of 50 to 150 per cent which is in agreement with the results of other workers.

Kensler *et al.* (181) have determined the activities of several enzymes in the livers of rats fed diets containing the noncarcinogenic 2-methyl-4-dimethylaminoazobenzene. Increased activities were noted for succinoxidase and choline oxidase which could be attributed to the increased mitochondrial counts that accompany the administration of this azo dye (173). In contrast to succinoxidase and choline oxidase, neither dimethylthetin-homocysteine

transmethylase nor the metabolism of 4-DAB by liver slices was increased when this inactive dye was fed.

Feeding of *o*-aminoazotoluene to mice, and of 4-DAB to rats, abolished the fatty acid oxidation of the liver. Fumaric, succinic, and α -hydroxycaproic acids enhanced fatty acid oxidation and restored the capacity of the livers damaged by azo dye feeding to oxidize fatty acids [Waterman *et al.* (182)]. Bernheim *et al.* (183) observed that several carcinogens inhibited the oxidation of skin fatty acids. Since oxidized fatty acids are more effective inhibitors of certain enzymes, the authors theorized that carcinogens, by inhibiting peroxide formation, may produce conditions favorable for growth. The activity of asparaginase in livers of rats fed 4-DAB was lowered as long as the dye feeding continued (184). If the animals were returned to dye-free diets before liver tumors were induced, the asparaginase returned to its normal value. Mori *et al.* (185) have investigated the effect of 4-DAB on catalase, and Siebert *et al.* (186) found that this azo dye inhibits liver cathepsin. 2-Acetamidofluorene and benzpyrene did not inhibit this latter enzyme. According to Mills & Wood (187) benzpyrene, previously stored in the dark, had no effect on the activity of urease. The same compound, when exposed to ordinary light, inhibited urease. This inhibitory action probably involved sulfhydryl groups since the addition of cysteine prevented this effect of benzpyrene on urease. Further studies on the role of sulfhydryl groups in carcinogenesis have been carried out by Wood & Kraynak (188). Intravenous administration of colloidal dispersions of benzpyrene in rabbits and dogs produced a decrease in the plasma sulfhydryl content. This effect was not produced by the administration of anthracene. Mori and associates (189) found that uricase of rat liver increased in the early stages of 4-DAB carcinogenesis and decreased somewhat when hepatomas were present. The uricase activity of the hepatoma was approximately 1/4 that of liver. These investigators (190) have also studied the iron content of the livers of rats fed diets containing 4-DAB. This same azo compound had no effect on the nicotinamide of either the liver or spleen of rats (191). Electrophoretic studies on the soluble proteins of cellular fractions from regenerating rat liver, from hepatomas, and from livers of rats fed diets containing 4-DAB or 3'Me-4-DAB have been completed by de Lamirande *et al.* (192) and Eldredge & Luck (193).

THE CHEMISTRY AND METABOLISM OF TUMORS

Amino acids, proteins, nucleic acids.—The relation of D-amino acids to the composition and metabolism of tumors has been reviewed by Shiba (194). Attention is called to the review of Miller (195) concerning the possible occurrence of D-amino acids in tumors. Wiltshire (196) determined the relative amounts of D-glutamic acids in several tumors. Values of from 0 to 6 per cent of the total glutamic acid were found for the D-isomer; however, this was not more than would be formed by inversion of the L-isomer during

hydrolysis. Many of the normal tissues studied also contained up to 6 per cent of the D-form. Boulanger & Osteux (197) incubated homogenates of intestinal mucosa with either pyruvic acid or oxaloacetic acid and obtained small amounts of D-amino acid (probably D-alanine). Considerably more of the D-amino acid was obtained when epithelioma tissues were incubated. The significance, if any, of D-amino acids in the metabolism or composition of tumors still awaits some unequivocal results.

Determinations of the free amino acids in Jensen sarcoma of rats have been completed by Di Bella (198). The tumor tissues contained more free cysteine and methionine and somewhat less lysine than normal tissues. Kit & Awapara (199) made quantitative measurements of the free amino acids of several tumors. Lymphosarcomas had a high content of alanine, glycine, and proline compared with normal tissue. Lower values were observed for aspartic and glutamic acid and ethanolamine phosphoric ester in the tumor. All of the lymphatic tumors had active transaminases. Yoshida sarcoma cells, according to Zamecnik (200), were capable of incorporating DL-leucine-1- C^{14} into protein under anaerobic conditions when incubated in an inorganic salt medium with added glucose. Addition of arsenate stimulated glycolysis but inhibited incorporation of the amino acid; dinitrophenol had no effect on either process. Further investigations on toxohormone have been carried out by Fukuoka & Nakahara (201). This substance, a polypeptide, is assayed by its depressing effect on liver catalase. Toxohormone increases in concentration in tumor tissues following the injection of protein hydrolysates into the tumor-bearing host. Eight amino acids were responsible for this effect: alanine, proline, aspartic acid, arginine, phenylalanine, lysine, leucine, and glutamic acid. These findings were interpreted as meaning that these amino acids are constituents of the toxohormone. Further confirmatory results will prove interesting if it can be demonstrated that amino acids injected into tumor-bearing animals are selectively utilized in the synthesis of a polypeptide substance. Labeled amino acids could be used to advantage in studying the biosynthesis of this substance.

The incorporation of methionine into proteins of Jensen sarcoma was reduced by administration to the tumor-bearing rats of 2,4-dinitrophenol, sodium pyrophosphate, or adenylyl pyrophosphate. X-radiation applied to the tumor, while reducing the uptake of P^{32} by the DNA, did not affect the uptake of methionine into the tumor proteins or P^{32} incorporation by the RNA fraction [Holmes & Mee (202)]. Vermund *et al.* (203) found that x-radiation inhibited P^{32} incorporation into the DNA of mouse mammary carcinomas, but was without effect on the incorporation of P^{32} into RNA, phospholipids, or "phosphoproteins." Malkin & Greenberg (204) have also observed that the addition of 2,4-dinitrophenol or nitrogen mustard to Ehrlich ascites tumor cells inhibited the incorporation of radioglycine into both the tumor proteins and ribonucleic acid. Evidence has been obtained by Miller *et al.* (205) for the existence of a hepatic factor that is essential for maximal

protein synthesis by the Walker rat tumor. Shack & Thompson (206) have described a procedure by which most of the DNA of mouse lymphosarcoma can be isolated as the nucleohistone. Viscosity, solubility, and other physical studies were carried out on this nucleohistone preparation. In addition, the DNA obtained from the lymphoma was also subjected to a variety of physical-chemical studies. The behavior of this tumor nucleic acid preparation was quite similar to that of the desoxypentose nucleate of calf thymus (207). Evidence has been obtained by Kennedy (208) for a metabolically active "phosphoprotein" in Ehrlich ascites tumor cells. The cells were incubated with orthophosphate- P^{32} and the acid-insoluble "phosphoprotein" fraction was isolated. Following prolonged hydrolysis in 2N HCl, the radioactivity was brought into solution but was still in bound form, suggesting that the high specific activity of this fraction was not attributable to adsorbed inorganic phosphate. Cornatzer *et al.* (209), employing P^{32} , have found a significant increase in phospholipid and protein-bound phosphorus in rabbit papilloma as compared with normal skin. From the relative specific activities obtained it was concluded that phospholipid synthesis was greater than was the synthesis of the protein-bound phosphorus fraction in the papilloma. It should be pointed out that the latter fraction includes DNA, both nuclear and cytoplasmic RNA, and "phosphoprotein." The activities of each of these component fractions, as measured by radioisotope uptake, are different and the activity of any given component may also vary from one tissue to another. The phospholipids also constitute a complex mixture. Individual components, instead of these complex mixtures, should be investigated to ascertain their possible role in the process of normal or malignant growth.

Additional information on the incorporation of C^{14} -labeled glycine and P^{32} into purines and proteins of normal and neoplastic tissues is provided by the investigations of LePage *et al.* (210, 211). The nucleic acid metabolism of the tumors studied, appeared to be qualitatively similar, but quantitatively more rapid than that of normal liver. The addition of the antibiotic isomer of chloroamphenicol to ascites tumor cells *in vitro* inhibited the incorporation of glycine-2- C^{14} into the proteins and nucleic acids whereas the inactive isomer inhibited the incorporation into purines but not into proteins. In lymphosarcoma cells both isomers were equally effective in inhibiting the incorporation of the C^{14} into proteins and nucleic acids (212). Mandel *et al.* (213, 214, 215) have established that labeled 4-amino-5-imidazolecarboxamide is a purine precursor in sarcoma 37 in mice. Guanine-4- C^{14} , injected intraperitoneally into mice bearing the tumor, was incorporated to a small extent into the nucleic acids of the liver and other internal organs but not into the nucleic acids of the sarcoma. The activity of the guanine from the liver was higher than that of the corresponding adenine fractions; however, there was definite activity in the adenine fraction of the RNA. These results provide evidence for the conversion of guanine to adenine in mouse tissues. Similar findings have already been reported for *L. casei* and *Tetrahymena geleii*.

8-Azoguanine did not alter the incorporation of either guanine or 4-amino-5-imidazolecarboxamide into the nucleic acids of the tumor or the liver.

Levy *et al.* (216) determined the DNA and the RNA of tumor cells following the inoculation of DBA mice with ascites thymoma. The DNA and also the RNA per cell showed a cyclic rise and fall as the ascites tumor developed. According to Menten *et al.* (217) the DNA phosphorus content per splenic lymphocyte of normal mouse spleens varies between 0.37×10^{-9} and 0.57×10^{-9} mg. and the RNA phosphorus per lymphocyte varies between 0.16×10^{-9} and 0.26×10^{-9} mg. The DNA phosphorus of splenic lymphocytes from leukemic mice varied from 0.66 to 0.72×10^{-9} mg. and the RNA phosphorus from 0.29 to 0.49×10^{-9} mg. Possible reasons for the higher values in the leukemic mice are discussed by the authors. The relative amounts of DNA per nucleus in individual nuclei of several different tumor tissues were measured by Bader (218) employing absorption microspectrophotometric methods. The DNA per cell was distributed into classes in a geometric progression of 1:2:4. Tumor tissues exhibited increases in the DNA per cell as compared with the normal control tissues. The increase of DNA classes in tumor tissues was considered to be associated with increased mitosis and polyploidy and not to be a result of disturbances in nucleic acid synthesis. Since mitosis and polyploidy are present in a number of normal growing tissues, Bader concluded that DNA change alone cannot be considered a causative factor in tumor formation.

From results reported by Chargaff & Lipshitz (219) the purine and pyrimidine concentrations of DNA of normal human liver and of carcinomatous human liver are almost identical. These investigators ascertained that the sugar from both tissues was 2-desoxyribose. Woodhouse (220) determined the purines and pyrimidines of DNA by chromatography and ultraviolet absorption for a group of experimental tumors and human carcinomas. The adenine/guanine and thymine/cytosine ratios were greater than 1. Certain differences in the purine and pyrimidine content of the tumors were noted when compared with normal tissues. However, there was no constant pattern that would distinguish the DNA of the tumor tissues.

Enzymes: oxidative.—Investigations and discussions have continued as to the oxidative capacities of normal and neoplastic tissues. Wenner, Dunn & Weinhouse (221, 222) have made further studies on the oxidation of components of the citric acid cycle by the mitochondria of tumor tissues. Oxidation of these components occurs readily when the system is fortified with DPN. The interesting suggestion was made that mitochondria of neoplastic tissues do not bind DPN as strongly as certain normal tissues, and, therefore, a higher concentration of the coenzyme may be present in the soluble portion of the cytoplasm. Since the glycolytic enzymes are in this portion, it would be expected that glycolysis would be higher in such tumor cells. In studies involving the malic system, Potter (223) obtained Q_{10} values of 114, 103, and 81 for heart, liver, and kidney, respectively. Results obtained from

subsequent investigations involving aerobic glycolysis in homogenates provided values of 38, 18, and 23 for the same tissues (224). The authors suggested that the malic system was not limited by DPN-cytochrome-*c* reductase or that in an aerobic glycolytic system the triosephosphate dehydrogenase was unable to saturate the cytochrome reductase capacity in these tissues. Reif *et al.* (224) pointed out that tumor tissues, regardless of the test system, never exceeded an oxidative rate of Q_{O_2} 20 to 25, while the oxidation capacities of certain normal tissues far exceeds these values. The low mitochondrial content of tumors, observed by several investigators and confirmed recently by Allard and associates (225), would account for the low oxidative capacity of tumor tissues. A system for the aerobic glycolysis of fructose-1,6-diphosphate was established by Reif *et al.* (224). High values for certain normal tissues to a lower range of values for tumor tissues were observed. The addition of brilliant cresyl blue doubled the O_2 uptake of tumor tissues while no effect was observed on several normal tissues. This was attributed to a limited capacity of the DPN-cytochrome-*c* reductase in the tumors. Tumor tissue homogenates produced lactic acid from glucose while normal tissues were unable to produce lactic acid from glucose in the absence of the substrates.

The carbohydrate metabolism of leukocytes has been ably reviewed by Beck & Valentine (226). Young & Taylor (227) have studied the factors influencing the utilization of glucose by egg-cultivated mouse tumor tissues and by the 5½-day chick embryo. The aerobic and anaerobic glycolysis of mouse ascites tumor cells have been investigated by Tiedemann (228). A considerable oxidation of glucose-6-phosphate and 6-phosphogluconate by ascites tumor extracts was observed by Williams-Ashman (229). The dehydrogenases for these components are specific for triphosphopyridine nucleotide. Glucose did not reduce the pyridine nucleotides in these studies, suggesting that glucose dehydrogenase is not present in the ascites tumor cells. Brin (230) found that glycolyzing mouse ascites tumor cells produced L(+)-lactic acid, which confirms earlier results obtained by Warburg that L(+)-lactate is produced by glycolyzing tumor tissues. Lindberg *et al.* (231) measured the respiration and phosphorylation and found both to be very low in Ehrlich ascites tumor mitochondria. With several substrates the rate of phosphorus uptake varied from 0.03 to 0.05 μ moles P per mg. N per min. Values of approximately two μ moles have been obtained for mouse liver and other normal tissues. Low P/O values were obtained for the tumor mitochondria with most substrates; only succinate gave a normal ratio. Addition of RNA or DNA lowered the respiration of Ehrlich's adenocarcinoma, according to Deotto (232).

Burk *et al.* (233, 234) have carried out further studies on the metabolism of melanoma tissues. Both brain and melanoma slices displayed similar $Q_{CO_2}^{O_2}$. Magnesium and phenol both increased the $Q_{CO_2}^{N_2}$ of brain but had slight effect on that of melanoma. The $Q_{CO_2}^{N_2}$ of brain slices was not increased by

insulin, while that of melanoma was increased by 40 per cent. These investigators discuss in some detail the aerobic and anaerobic metabolism of these two tissues. Also mentioned was the possibility that mitochondria may act as genetic determiners in mammalian tissues. Abnormalities in the function or the composition of mitochondria may certainly represent the cause of self-perpetuating cellular abnormalities. However, considerable research will be required to establish the role of the mitochondria as well as the cell nucleus in the carcinogenic process and the subsequent growth of the newly formed tumor cells. Roberts & White (235) found that adrenalectomy, ACTH, or adrenal cortical extract had no effect on the respiration or aerobic glycolytic activity of lymphoid tissues from the surviving normal rat, i.e., spleen and thymus. In contrast, the aerobic utilization of glucose by lymphosarcoma tissue *in vitro* was elevated by previous treatment of the animal with ACTH.

The effects of 2,4-dinitrophenol and fluoride on the coupled oxidative phosphorylation systems of liver, kidney, and of several different tumors have been investigated by Siekevitz & Potter (236). Clowes & Keltch (237) have noted a preponderance of glycolytic over oxidative phosphorylation in Walker 256 tumor. Single subcutaneous injections of tumor-damaging agents such as arsenicals, phenazines, and colchicine in mice bearing sarcoma 37 and other tumors produced a decrease in cytochrome oxidase activity in the tumor tissue homogenates (238). Goddard & Seligman (239) have developed histochemical techniques for demonstration of succinic dehydrogenase in rat hepatoma. The "large" microsome fraction of mammary cells of mice contains appreciable amounts of succinoxidase, according to Dmochowski & Strickland (240). These authors are not of the opinion that this activity represented mitochondrial contamination. No difference in succinoxidase content or its distribution could be detected between normal mammary tissue and mammary tumors of mice.

Investigations of lipid synthesis in neoplastic tissues have revealed that acetate and glucose carbon can be utilized for synthesis of fatty acids. The rate of the process is probably too slow, according to Medes *et al.* (241), to supply the lipid requirements of the rapidly growing tumor, and the tumor must obtain its lipid preformed from the host. In subsequent studies these investigators (242) demonstrated that slices of transplanted tumors oxidized C^{14} -labeled fatty acids to CO_2 . The rate of oxidation was generally lower than that observed for liver slices, especially with the short-chain fatty acids. Busch & Potter (243, 244) have studied the metabolism of acetate- $1-C^{14}$ in the tissues of tumor-bearing rats. Malonate was used as a block in order to accumulate a pool of succinate. A maximal incorporation of C^{14} in the normal tissues, as indicated by the activity of the succinate pool, was reached 2 to 4 min. after the intraperitoneal injection of the acetate. Most of the radioactivity in Flexner-Jobling tumor remained volatile for 30 min. after the injection of the precursor. The utilization of acetate, which readily

occurs in most normal tissues, was greatly diminished in the tumors studied. These findings and previous observations by these investigators of lowered citrate in the tumor of fluoroacetate-treated animals were interpreted to mean that the tumor is more dependent upon blood-borne intermediates of the Krebs cycle than upon its internal sources. The above investigations should be extended to nontumor-bearing rats. If the tumor is dependent upon preformed metabolites of the Krebs cycle, it can not be assumed that the metabolic activities of the tissues from normal and tumor-bearing rats would be identical.

Other enzymes.—Brain tumors, comprised predominantly of glia cells, contained approximately the same amino oxidase and cholinesterase activity as that in normal brain areas (245). Maver *et al.* (246) have determined the catheptic activities of the nuclear fraction of normal and neoplastic tissues of the rat. Alkaline phosphatase activities of epidermoid carcinoma of the cervix before and after x-radiation, have been measured by Rev *et al.* (247). Sacerdote de Lustig & Sacerdote (248) have made studies on the activity of this enzyme in intracellular components of various normal and tumor tissue cultures. Shack (249) has found that the desoxyribonuclease of transplantable lymphoma of strain A mice has an optimum at pH 5 and is not activated by Mg^{++} or inhibited by citrate. The enzyme from serum of this strain of mice differs from that of the tumor by having a maximal activity over the pH range of 7 to 8. This serum enzyme is also activated by Mg^{++} and completely inhibited by citrate. Studies of liver extracts indicated that two depolymerases were present, presumably those that occur in the serum and in the tumor.

Adrenal carcinomas in castrate CE mice and interstitial cell tumors in male mice of the BALBC strain showed 3β -ol-dehydrogenase activity proportional to the androgenic function of the tissue (250). This enzyme catalyzes the oxidation of certain Δ^4 -unsaturated steroids containing a 3β -hydroxy group to the α,β -unsaturated Δ^4 -3-ketone. Charalampous & Mueller (251) have studied the enzymatic formation of erythrulose phosphate by the condensation of C^{14} -labeled formaldehyde with triosephosphate. Rat liver has a high activity in this respect, while most experimental tumors were almost devoid of activity. The enzyme responsible for this synthesis is different from the Meyerhof-Lohmann aldolase since the aldolase activity of most tumors, as well as of rat livers, is quite high. The inability of tumors to catalyze formaldehyde means that the enzyme is lacking or that an inhibitor is present. A new method for estimating acid phosphatase has been described by Fishman & Lerner (252). L-tartrate is added, which inhibits the acid phosphatase of prostatic origin. Any appreciable value obtained then reflects phosphatase of tumor origin. The normal distribution of this serum enzyme activity in older men and women was 0.0 to 0.5 units per 100 ml. of serum. Higher values were observed in cases of untreated cancer of the prostate (253).

Other properties of tumors.—Wollman *et al.* (254, 255) have studied the radioiodine uptake by transplantable tumors of the thyroid gland in mice. The ascorbic acid content of Yoshida sarcoma and of other tissues has been determined during the development of the tumor by Shibata (256). Caruthers & Suntzeff (257) have determined the nicotinamide content of normal and malignant tissues. These same workers have also developed a polarographic method for measuring pyridine nucleotides of tissues. The average value obtained from a group of tumors was 120 $\mu\text{g./gm.}$, while normal tissues varied from 200 (spleen, lung) to approximately 500 $\mu\text{g./gm.}$ for liver and kidney (258). Shapiro *et al.* (259) have measured the concentration of vitamin B₆ and other constituents of the tumor and of several tissues of the host as a basis for the selection of chemotherapeutic agents.

Further information regarding the effects of adrenalectomy and hypophysectomy on tumor growth has been supplied by Talalay *et al.* (260). ACTH-secreting transplantable pituitary tumors have been described by Furth *et al.* (261). There was no evidence of stimulation of any endocrine organ other than the adrenal. A human adrenal tumor was shown, in organ perfusion studies, to utilize sodium acetate-1-C¹⁴ as substrate for the synthesis of cholesterol [Smith & Werthessen (262)]. Evidence for the synthesis of cobalamin (vitamin B₁₂) by spontaneous mammary tumors has been obtained by Woolley (263). When this tumor was transplanted, however, there was no further evidence of cobalamin synthesis. The effect of citrovorum factor and antagonists on leukemia and other neoplasias has been ably reviewed by Baumann (264). Patt *et al.* (265) and Klein & Revesz (266) have studied the growth characteristics of ascites tumors. The growth and regression of the Rous sarcoma is associated with an age factor according to Freire *et al.* (267). Warren *et al.* (268) have prepared a mucopolysaccharide from ascites mouse tumor.

THE EFFECT OF TUMORS ON THE FUNCTION AND COMPOSITION OF BODY TISSUES AND ORGANS

From the results obtained by many investigators in recent years there is mounting evidence that tumors are responsible for considerable alteration in the composition and metabolic activities of the normal tissues of the host. Perhaps this is to be expected; however, the growth and survival of autonomous tumor tissues may depend to a considerable extent on normal tissue function. It is not possible to review the findings of investigators over the past few years which have established that the presence of tumors affects the nucleic acid metabolism of the normal tissues. As mentioned previously, Medes *et al.* (241) believe that the rate of lipid synthesis in neoplastic tissues is probably too slow to supply the lipid requirements of the growing tumor. Busch & Potter (243, 244) also postulated that the tumor is more dependent upon blood-borne intermediates of the Krebs cycle than upon its internal sources. The mechanism by which tumor tissues alter the function

or composition of normal tissues should be investigated in order to determine the extent of tumor dependence upon normal function.

Amino acids, proteins, nitrogen balance.—Bolker (269) has investigated nitrogen balance in patients with malignant disease. In late carcinoma cases, according to Esser, Heinzler & Wild (270), the concentration of serum albumin is decreased while the concentrations of various serum globulin fractions are increased. The location of the carcinoma or the absence or presence of metastases had no influence on the serum protein distribution. Several reports (271, 272) have appeared on the chemical and physical nature of the serum proteins in multiple myeloma. Putnam (273) has found that normal γ -globulin contains aspartic and glutamic acids as the major N-terminal groups, whereas the γ -globulin of myeloma patients contains neither amino acid in this position. This is one of the first demonstrations of a difference in the chemical structure of a serum protein elaborated during disease. Kanzow (274), using paper electrophoresis, has analyzed the serum proteins of patients with chronic myeloid or lymphatic leukemia. Differences in the amino acid composition of the serum proteins of normal and tumor-bearing rats have been reported in a preliminary publication by Schultz *et al.* (275). Di Bella (198) did not observe any differences in the free amino acid content of the tissues of normal rats or of rats with Jensen sarcoma. According to Rumsfeld & Baumann (276) rats bearing tumors induced by feeding of diets containing azo dyes excreted more protein than normal rats. This effect was peculiar to hepatomas, since no increase in proteinuria was observed in rats bearing other types of tumors. Albaum *et al.* (277) have found that during tumor growth in mice, as the concentration of blood nucleotide decreases, the concentration of tumor nucleotides increases. Eight days after the tumor implantation the concentration of tumor nucleotides undergoes a rapid fall and the blood level approaches the normal level.

Enzymes.—Pascaud (278) has noted a decrease in the liver lecithinase in rats with hepatomas or grafted Guerin epithelioma. No significant differences in desoxyribonuclease activity were detected in serum from normal persons or patients with malignant diseases [Kurnick (279)]. Black & Speer (280) have measured the relative dehydrogenase activity of kidney and liver *in vitro* from several strains of normal and tumor-bearing mice.

Additional information on the depression of liver catalase in tumor-bearing animals has appeared from several sources (201, 281, 282, 283). Lucké *et al.* (283) concluded that the substance responsible for this depression was transferable across a parabiotic union. No significant difference was observed in blood catalase activity between normal and cancer patients [Papadodoulou (284)]. Feinstein & Ballin (285) have found that the blood cells of tumor-bearing rats were free of an inhibitor for cysteine-requiring carboxypeptidase. However, the inhibitor was present in the cells of normal rats and rabbits. In normal persons and in cases of nonmalignant disease the blood titer for this inhibitor was high; in cases of malignant disease the titer was decreased

(286). The test was not considered specific enough for the clinical detection of cancer.

Effect on other tissues components.—Bach & Maw (287) have observed that sarcomatous rats excreted 8 to 10 times more creatine than did normal rats. These authors concluded that this excessive excretion was the result of an increased endogenous synthesis of creatine associated with tumor growth. Leukemic mice also excreted more creatine than did the controls, according to Dinning *et al.* (288), thus indicating that creatine and methyl groups are involved in the formation of leucocytes.

During the accelerative phase of growth of lymphosarcoma in rats, Shacter *et al.* (289) noted a decrease in the sulfhydryl content of the plasma. As the tumors regressed, the plasma sulfhydryl levels returned to normal. Administration of cortisone to the tumor-bearing rats delayed the accelerative phase of tumor growth and also the plasma sulfhydryl decrease for a few days. These workers suggested that adrenal hormones exert their inhibitory effect on tumor growth by inhibiting the utilization of sulfhydryl groups essential for the tissue proliferation. Myelokentric acid and lymphokentric acid are substances which are obtained from the urine or serum of leukemic patients and which produce characteristic cellular changes when injected into guinea pigs. These acids have been concentrated and further studied by Turner and co-workers (290). Rauramo & Wallgren (291) have found that the serum copper levels were elevated in cases of cancer of the uterus. A clinical case of primary carcinoma of the liver accompanied by ethanolaminuria has been reported by Dent & Walshe (292). The significance of this obscure metabolic defect is discussed. The blood volume and water balance in patients with advanced neoplastic diseases have been studied by Kelly *et al.* (293). Boyd *et al.* (294) analyzed the carcasses, skeletal muscle, and testicles of tumor-bearing rats for water content and for several lipid components. Generally, shifts in concentration of these components of the normal tissues were toward the level of the tumor component.

SUMMARY

Several new experimental methods for inducing cancer may be added to the already long list. The mechanism involved in the production of tumors by the embedding of plastic substances should be investigated. Prolonged administration of either *o*-aminoazotoluene or acetylaminofluorene resulted in the formation of tumors in dogs. Tannic acid appeared to enhance the hepatocarcinogenic action of acetylaminofluorene. Thyroid tumors were produced by the administration of I^{131} alone or with methylthiouracil. Rats maintained on low iodine diets for prolonged periods developed a high incidence of thyroid and pituitary tumors. Hepatic tumors appeared in rats fed diets containing ethionine for 50 to 150 days.

Knowledge of the basic steps in the process of carcinogenesis is required. We now have many methods at our disposal for the induction of cancer.

Only fragmentary data are available concerning the changes that occur as neoplastic cells originate. While it would appear improbable that all of the methods of cancer induction work through a common pathway, it is possible that they may all have certain features in common. Changes in the mitochondrial composition or activity may be an important feature of carcinogenesis. Carcinogenic agents do alter the enzymatic activities of mitochondria, as well as of other fractions of the cell. However, these changes are more quantitative than qualitative. It has been thought for a long time that carcinogenic agents alter the nuclear portion of living cells. There is still no conclusive evidence that the desoxyribonucleoproteins of normal or of "pre-cancerous" tissues differ in composition. However, irreversible changes in chromosomes and genes must represent a final phase of the carcinogenic sequence. The biochemical nature of these changes or the reactions that lead to these changes must still be established. Hormonal factors are undoubtedly involved in enhancing or modifying the action of carcinogenic agents. Removal of the pituitary protects rats against the carcinogenic action of azo compounds. Tumors will develop, however, in hypophysectomized rats exposed to the carcinogenic hydrocarbons and other methods of cancer induction. Adrenal function may be an important factor in carcinogenesis. This approach is complicated by the adrenal accessory tissue present in many animals. The availability of more corticoid substances along with improved chromatographic techniques for investigating the metabolism of the steroids will contribute greatly to this phase of cancer investigation. The increase in the incidence of lung cancer in man should stimulate new studies of the environmental factors that are apparently involved, as well as of the mechanisms of lung carcinogenesis.

There is further evidence that dietary factors are involved in cancer induction. Diets containing bentonite, fed to mice, resulted in liver damage and eventually of hepatomas. The bentonite presumably removes choline from the intestinal contents. Prolonged iodine deficiency results in thyroid neoplasms. Ethionine, a methionine antagonist, will promote tumor formation. There are further data to suggest that underfeeding or restriction of calories will alter carcinogenesis by increasing the latent period of tumor formation. Underfeeding also retards the rate of growth of established tumors. There is no evidence of any nutritional deficiency that will inhibit tumor growth specifically. Actually, tumors appear to survive and grow when the normal tissues are severely restricted by the diet.

Studies on the metabolism and composition of tumor cells have still not revealed any characteristic compositional or metabolic pattern. Investigations have continued on the oxidative capacities of normal and neoplastic tissues. The generally lowered oxidative activities of tumors appear to be of a more quantitative than qualitative nature. Tumor tissues are deficient in certain enzymes. The significance of these deficiencies in tumor metabolism must still be ascertained. There is increasing evidence that neoplastic tissues

must rely on the normal tissues for fatty acids and for components of the Krebs cycle. The presence of tumors also appears to disturb the nucleic acid metabolism of many normal tissues in the host animal. This relation between tumor and the normal host tissues appears to be worthy of further extensive study. The mechanism of the formation of tumors and the compositional or metabolic patterns by which neoplastic tissues differ from normal tissues still challenge the biochemist.

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THE BIOCHEMISTRY OF MUSCLE¹

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Introduction.—The present review covers the period 1952 to 1953, with occasional reiteration of earlier work. A broad coverage is attempted, but studies on ion distribution, and on certain phases of metabolism covered in other chapters in this volume, have been omitted. Work done in previous years is easily accessible through the almost simultaneous appearance of several reviews (330 to 333) and monographs (334, 335, 336). The transactions of a few symposia and discussions have also been published (113, 327, 328, 329).

Muscle constituents and analytical procedures.—Chromatographic separation of sugar phosphates (as boric acid complexes) on an anion exchange resin has been achieved (1). In combination with the already available separation of adenosine-5-polyphosphates (2), this allows the analysis of complex tissue extracts. The separation of AMP, ADP, and ATP¹ has been applied to muscle extracts or enzyme digests by several authors (120, 151, 152, 240); inosine polyphosphates can likewise be separated in this manner (7, 120, 240). Several systems for chromatography of nucleotides on paper have been described; the use of an ammonia-propanol-water system seems to give the best results (71, 242). Among enzymatic methods of analysis, the older method of Kalkar remains important (applications: 119, 123, 152, 154), either as such, or with hexokinase instead of apyrase. However, small amounts of ADP are not accurately determined in this manner; for this purpose, the method of Kornberg & Pricer (3) is recommended (154). A scheme for the analysis of several phosphorylated compounds was developed by Slater (4, 5). The light emitted by a firefly extract can be utilized to determine ATP and other substances (6).

The important discovery of the role of IDP and ITP¹ in metabolism (7), and of the transphosphorylation between adenosine- and inosine- or uridine-phosphates (8) may have consequences for the study of muscle. The possible occurrence in muscle of adenosine-5-tetraphosphate (9) and of uridine triphosphate (10) has been announced. A new adenosine derivative may occur in hearts in systole (121), but needs further characterization. Electrochemical properties of adenine nucleotides (11, 12) and the existence of calcium complexes of ATP (13) have been described.

New phosphagens have been discovered in marine worms (14), namely phosphoglycocyanine (synthesis: 15), and phosphotaurocyanine. The former is acted upon by creatine phosphokinase from mammalian muscle (15). The method of Berenblum & Chain (15a) has been adapted for the separate

¹ The following abbreviations are used: AMP for adenosinemonophosphate; ADP for adenosinediphosphate; ATP for adenosinetriphosphate; IDP for inosinediphosphate; ITP for inosinetriphosphate.

determination of phosphate and phosphocreatine (16), while free creatine (17, 18) and phosphocreatine (18) have been determined in muscle with the diacetyl reaction.

Several papers on tissue amino acids contain data on muscle (19, 20, 21). The increase in free amino acids caused by cortisone is greatest in muscle, but glutamic acid is not affected (21). Schmidt *et al.* (22) determined carnosine plus anserine in terms of bound β -alanine, of which there is about 1 mg. per gm. fresh muscle, but none in the heart.

Dreyfus & Schapira have extended their work on the iron content of muscle (reviewed in 24). Part of the nonheme iron is not extractable with acid; this fraction is typical for muscle and appears bound to myosin (23). In fatigue there is an increased turnover of this bound iron (97); other studies deal with its metabolism in atrophy (98) and in ischemia (99). Additional studies on muscle glycogen have been published (25, 26).

Soluble proteins.—Proteins extractable by dilute salt are presumably of sarcoplasmic origin. Further electrophoretic characterizations of this fraction (cf. 191) have been described (27). It is stated that after grinding with sand, a viscous solution is obtained at ionic strength $\mu=0.13$, from which after several hours myosin precipitates. It is concluded from this that myosin is an artifact; however, the observation has been contradicted (28). The distribution of the soluble proteins in different types of vertebrate muscles has been studied (29, 30), and data are available on fish muscle (31, 32, 33).

Myoglobin is increased in muscle as a result of persistent exercise (34), and its turnover in muscle has been studied (35). It has been isolated from fish (36). Fetal myoglobin differs from the adult form (37). A number of myogen fractions, including several enzymes, have been crystallized (38).

Enzymes and metabolism.—Glycolysis has been reviewed in one of Meyerhof's last papers (39). The inhibition of glycolysis by glyceraldehyde (40) and the effects of carnosine (60, 60a), of sulfhydryl reagents (41) and other substances (42) have been studied. The phosphorylase equilibrium (43) and specificity (43a) have been reinvestigated. A phosphorylase has been demonstrated in *Ascaris* (44). Hexokinase activity has been determined in rat tissues (45); one may estimate that in muscle it occurs in large excess over the requirement to cover resting metabolism by a steady uptake of blood glucose, but that it does not suffice for activity metabolism in which glycogen will be drawn upon. A hexokinase activator occurs in muscle (46). Carbohydrate metabolism and phosphorus compounds in smooth muscle were studied (47, 47a). The stability of some enzymes during cold storage of muscle has been determined (47b).

Rat diaphragm and other muscle metabolizes hexosephosphates *in vitro*, reportedly by enzymes escaping from the tissue (48). In this system, α -tocopheryl phosphate is considered to inhibit phosphoglucumutase (49 to 52). The effect of insulin upon the isolated rat diaphragm can be explained in terms of a stimulation of hexokinase and an additional effect on the condensation of pyruvate with oxaloacetate (53). Insulin also increases the incorporation of

alanine (C^{14}) into diaphragm protein (54). The uptake of glucose, even anaerobically, is not limited by the available ATP (55). Cortisone inhibits glucose uptake without affecting glycogen formation or lactate production (56).

The turnover of P^{32} is greater in flexor than in extensor muscles, reflecting the predominance of tonus fibers in the latter (57). The uptake of P^{32} into muscle or cardiac ATP is more rapid in the terminal than in the second phosphate group (58, 123). Criticism has been brought forward (59) against the correction for extracellular phosphate in earlier tracer work in muscle.

Isolated diaphragm utilizes serum lipids. This utilization is suppressed by glucose, pyruvate, and acetoacetate (61). Octanoate at low concentrations stimulates the O_2 consumption of diaphragm (61a). Fixation of CO_2 is described for pig heart (62) and pigeon breast muscle (63).

A correlation has been sought between the activity of the cytochrome system and the amount of myoglobin present (64). In general, red muscles contain more of the oxidative enzymes, but there are some notable exceptions, which might increase if a wider variety of muscles were to be tested. Red muscles also have a higher capacity for aerobic phosphorylation, lower anaerobic glycolysis and adenosinetriphosphatase, and a lower store of energy-rich phosphate (65). Repeated injections of cytochrome- c are stated to increase the cytochrome oxidase in heart and kidney, but not in liver and muscle (66).

Enzymes involved in high-energy phosphate metabolism, not related to structure proteins.—The concept of phosphate bond energy has been scrutinized (67); the criticism goes too far, but deserves consideration to prevent a too colloquial use of the term. The thermodynamic instability of $\sim P$, in addition to resonance stabilization of the reaction products, is ascribed to an electrostatic effect in phosphopyruvate, ADP, and ATP (68). The mechanism of ATP-hydrolysis by lobster muscle has been investigated with H_2O^{18} . The isotope appears in the inorganic phosphate; the enzyme also catalyzes a transfer of O between phosphate and water (69). The free-energy effect associated with the hydrolysis of ATP has been ingeniously re-estimated (70); the value obtained is $-\Delta F = 9400$ cal. at pH 7.5, at unimolar activity; for concentrations prevailing in tissues, it may be as large as 13,000 to 16,000 cal.

The myokinase equilibrium has been investigated by paper chromatographic (71) and ion exchange separation (240) of the reaction products, with perfect agreement; $K = [ATP][AMP]/[ADP]^2 = 0.45$.

Lardy *et al.* (72, 73) have crystallized and characterized creatine phosphokinase from muscle. Roche *et al.* (74) have synthesized pyrophosphoryl choline; this is not split by muscle adenylpyrophosphatase, but is hydrolyzed by alkaline pyrophosphatase from autolyzed muscle.

Metabolism in rigor.—Earlier work in this field has been continued (75). The chief chemical events in development of rigor are as follows: (a) glycolysis starting at death, the rate depending on the pH region traversed; (b) fall of phosphocreatine when the pH reaches 6.8; (c) fall of ATP until it reaches 20 per cent of the resting level; after this, glycolysis and ATP breakdown diminish greatly. At $37^\circ C.$, but not at $17^\circ C.$, the muscle short-

ens considerably at 20 and 60 per cent of the phosphocreatine and ATP-levels, respectively. Some comparative data on rigor (76, 77) include observations on whale muscle which has a very high buffer capacity.

Changes during embryonic development.—In homogenized muscle of adult fowl the predominant adenosinetriphosphatase is of the Ca-activated type with pH optima near 6.5 and in the alkaline region; during development of the embryonic muscle, this enzyme increases relatively, while a soluble adenosinetriphosphatase decreases (78). In late embryonic development, there is a considerable increase of inorganic phosphate and especially of phosphocreatine and ATP in the muscles (79, cf. also 80); there is also a marked increase in glycogen (81). Csapo & Herrmann (82) find that in the early chick embryo there is practically no myosin or actomyosin; these reach levels of 5 mg. per gm. at 10 days, and 30 mg. at hatching, as compared with 60 mg. in the adult. The formation of myosin in the embryonic heart has been studied with immunological techniques (82a). For other studies on proteins see papers by Robinson (83, 83a).

Degeneration and dystrophy.—With histochemical techniques, the localization of cholinesterase in normal (84, 85) and degenerating (86, 87) muscle has been investigated. Specific acetylcholinesterase is restricted to the myoneural junction; no generalization seems possible as to the changes undergone after denervation. Several authors have determined enzymes or other materials during the degeneration following neurotomy, such as cytochrome oxidase (88, 88a), which decreases in parallel with succinic dehydrogenase after a transitory increase of the latter (88a); pyrophosphatase, which increases after neurotomy (no change after tenotomy), while adenosinetriphosphatase decreases (89); lipids, which decrease first and increase later, while cholesterol shows a rise only (90); hexokinase, which increases somewhat (91) (in this determination, adenosinetriphosphatase would be included); succinic dehydrogenase, which decreases [but this needs more clarification on account of the different types of enzyme (92)]; and phosphorylase, which is said to increase when functioning synthetically, but to decrease when phosphorylytic activity is measured (91). Since phosphorylase was determined in whole extracts, the antithesis between synthetic and lytic action might be attributable to other enzymes. These biochemical changes, when referred to wet or dry weight in an organ undergoing histological modification, cannot be quantitatively related to inherent changes in the muscular components themselves. Baldwin *et al.* (93), in a study on the creatine, P, K, and Mg in biopsy material of normal and dystrophic muscle, relate their analyses to the noncollagen nitrogen and find, on this basis, no change in dystrophic muscle. Dreyfus & Schapira (94), taking into account the increased fat and connective tissue, find a significant decrease of glycogenolytic activity and of phosphoglucomutase in myopathy; they also refer to a system of reference for chemical analysis of myopathic muscle and to decreases of aldolase and of phosphorylases *a* and *b* (95). Hexokinase is reported to increase after neurotomy, but to decrease in atrophied human muscle, according to measurements on the disap-

pearance of glucose in muscle, in which also, e.g., inhibitors may have an effect (96). In atrophy, as a result of inanition, vitamin E deficiency, motor nerve section, or immobilization, a fast electrophoretic component is augmented, while all other soluble proteins decrease; in human muscle atrophy, the results are different (100).

In muscular dystrophy, the decrease in concentration of plasma phosphate upon the injection of glucose is diminished (101). Increased serum aldolase (108) and ribosuria (109) have been reported to accompany myopathies. In vitamin-E-deficient animals, there is a more rapid incorporation of P^{32} into the inorganic P of muscles (102); the interpretation that the transformation into organic P is decreased requires direct experimental confirmation. Muscles of vitamin-E-deficient hamsters are reported to release less amino N *in vitro* (103). In deficient rabbits, creatinuria and a loss of muscle creatine precede any symptoms or observable histological changes, while simultaneously the concentration of creatine in liver, kidney, and blood increases and that of glycocyamine remains largely unaltered (104); in the same condition, muscle strips show increased respiration without change in R.Q. and glycolysis; with the onset of symptoms, glycolysis is increased (105). Evidence is presented that the liver is the major site of formation of both creatine and its precursors (106) and that creatinuria in dystrophy is caused by failure of newly formed creatine to enter the muscles (107).

It is reported that in muscular atrophy after neurotomy, the molecular length of myosin is slightly decreased, and the anisotropy increased (110, 111, compare 111a); among other possibilities, it is discussed whether atrophy may constitute a "molecular disease." However, the differences in length (determined by flow birefringence), particularly, are so small as to require confirmation by other methods. Also, the molecular lengths (about 2800 Å) are in excess of values determined otherwise (*vide infra*).

A review on experimental dystrophy (112), and the Proceedings of two conferences on muscle (113, 329), contain numerous references.

Smooth muscle.—Contrary to earlier findings on rabbit aorta, the adenosinetriphosphatase activity of extracts of dog coronary arteries is not affected by vasodilatory drugs (114); arteries contain unspecific cholinesterase (115). The tension developed by contracting uterus is controlled by ovarian hormones, in parallel with its actomyosin content (116); also the energy metabolism of the uterus is affected by the endocrines (117).

Myocardium.—An extensive study by Lorber (118) on the metabolism of the isolated heart confirmed the Starling and Visscher theory that failure is characterized by decreased efficiency at constant O_2 consumption. The metabolism of the human heart has been studied *in vivo* with catheterization techniques, with respect to the consumption of O_2 , and of glucose, lactate, and pyruvate; the three substrates could not completely account for the observed respiration (122). Greiner (119) reports analyses of phosphorylated compounds in the papillary muscle of the cat's heart; ATP, but not phosphocreatine, was decreased when the systolic force had appreciably declined in

phosphate-Ringer, and returned to normal when, under the influence of ouabain, the contractile force was restored; in anoxia, both ATP and phosphocreatine decreased. The total nucleotide content averaged $4.1 \mu\text{M}$ per gm. in fresh muscle. About $5 \mu\text{M}$ per gm. were found in whole mammalian myocardia, somewhat more in the left than in the right ventricles (120); anaerobically, nucleotides are lost by the tissue in the form of inosinic acid (120). *In vivo*, in diastole, all or most of the nucleotide occurs as ATP, but in systole (caused by immersion in liquid air or dry ice), $0.5 \mu\text{M}$ per gm. occurs as an unidentified adenine derivative, chromatographically different from AMP, ADP, or ATP (121). Phosphocreatine has now been shown to be more abundant in the heart than had often been believed and has been found to increase in failure, in keeping with the concept of interfered utilization of high energy phosphates in this condition (121a).

The incorporation of P^{32} into the inorganic and acid-labile phosphate of dog hearts has been studied with a needle biopsy technique (123, 124). If induced by exercise, increased cardiac work caused an elevated P^{32} uptake and an increased rate of isotope equilibration between the fractions; phosphocreatine attained a higher isotope activity than inorganic phosphate and total acid-soluble phosphate.

In invertebrate hearts, glycolysis produces other acids than lactic (125). Pantothenate deficiency causes a significant drop in cardiac coacetylase and a reduction of respiration and pyruvate consumption (126). L-Lactate is oxidized 3 to 5 times faster than D-lactate in duck hearts, but the total utilization of lactate is not greatly different for the two isomers (127).

Numerous publications on cardiac metabolism, and the effect of cardiac glycosides thereon, are primarily of pharmacological interest and will probably find suitable discussion by other reviewers. Effects of digitalis factors, *in vitro*, have been described; promotion of the contraction of actomyosin fibers made from compressed surface-spread layers (128), of actomyosin threads (129), and of glycerol-extracted muscle fibers (130) have also been reported. Other papers are concerned with the small increase of ATP-splitting by homogenates (131), stimulation of myosin-adenosinetriphosphatase but inhibition of total adenosinetriphosphatase activity in heart extracts (132), inhibition of adenylate deaminase in crude cardiac actin (133), depression of the extractibility of actin from heart muscle (134), and promotion of polymerization of actin (135). Several of these reports are in need of confirmation or further study. A water-soluble substance prepared from a lipid fraction of liver increases the contractility of a hypodynamic papillary muscle preparation (136). Corhormon activates the adenosinetriphosphatase breakdown in homogenates in proportion to the Mg-content of the system (137).

Hajdu & Szent-Györgyi (138, 139), ascribe the "staircase phenomenon" to a regulation of the contractile force by intracellular K; a concentration of K which is too high (as established in rest or low-frequency activity) depresses activity. The authors indicate that a favorable balance is maintained by a plasma steroid and is similarly influenced by digitalis glycosides (139). It

is stated that a diminution of potassium concentration by 3 m. eq. per liter increases the tension from zero to the maximal value (140).

Purkinje tissue.—The specific conductive tissue of the heart, which is of muscular nature, begins to be investigated biochemically. This tissue has a very high Na content (K:Na ratio, 0.59 in the atrioventricular bundle, 0.38 in the nodal tissues, as compared with about 1.8 in ventricular muscle), and the total ionic strength is far higher than in blood (141). The lipids of this tissue, and of other parts of the heart, have been analyzed (142). The myocardium contains mainly unspecific cholinesterase (143, 144), but Mommaerts *et al.* (145) report that the conductive tissues (including the moderator bands) contain a specific acetylcholinesterase in amounts comparable to those found in peripheral nerve.

Activity and metabolism.—Based on older theories of the mechanism of the Pasteur effect, the concept has arisen, that, because of the obligatory coupling between respiration (and glycolysis) and phosphorylation, the maximally possible rate of metabolism is limited by the utilization or waste of energy-rich phosphate. Three important papers have appeared in this field. Boyer *et al.* (146) find a three-fold increase in the O_2 consumption of heart particles upon addition of hexokinase and glucose. Lardy & Wellman (147) report that liver mitochondria with low adenosinetriphosphatase activity have a low respiration unless acceptors such as AMP or ADP, creatine with creatine phosphokinase, adenosinetriphosphatase (149a), or glucose with hexokinase are added; or in response to dinitrophenol. Siekevitz & Potter (148), in a similar system, obtained increased respiration by addition of a citrulline-synthesizing enzyme; the presumed dephosphorylation of ATP was not reflected in a lower ATP level in the suspension medium. The direct applicability of these results to muscular activity has not been investigated, but certain results of Biró & Szent-Györgyi (149) may be reinterpreted in this light.

The major question remaining in this field is whether the breakdown of ATP is an integral part of each twitch. Attempts to demonstrate this in the liquid air-induced contraction (121, 150, 151) are not convincing since the mechanism of this shortening is not well understood; for frog muscle (150, 151), the result could not be confirmed (152), possibly because of a different treatment of the control muscle. For a more direct approach to the problem, it is necessary, by rapid cooling, to interrupt the contracting muscle at an arbitrary stage. Preliminary experiments (153) suggested an ATP breakdown, and an extensive paper by Münch-Pedersen (152) makes such a claim in great detail. This must be criticized; on the one hand, greater accuracy is ascribed to the chromatographic analyses than seems possible, and the differences published are not convincing. On the other hand, by a wrong calculation the impression is created that the alleged difference is of sufficient magnitude to account for the initial heat, which is not the case. Current work with a high speed congelation technique has as yet failed to demonstrate any breakdown of ATP during contraction (154).

Intracellular organization of metabolic systems.—In the muscle cell, the

main system for ATP-utilization is formed by the contractile fibril (*vide infra*); besides these, particulate and soluble enzyme systems exist, comparable to those in other types of cells. The granules seem to be of two types: large interstitial particles and smaller spherical bodies. The former can be identified with mitochondria; for the latter, it may be advisable to adopt the term sarcosomes and so call all the particles collectively cytochondria (155), although "sarcosomes" originally included both types and other terminologies persist (163, 165). Since, as in other tissues, the cytochondria are the bearers of the oxidative-phosphorylative enzyme systems, there is a correlation between the profusion of granules and the preponderance of oxidative metabolism (156).

In rabbit skeletal muscle, which has a low oxidative metabolism, the sarcosomes have been studied by Perry (157, 158), mostly with respect to adenosinetriphosphatase (*vide infra*); the particles contain less nucleic acid than the microsomes of other tissues. The more active pigeon breast muscle is richer in mitochondria and sarcosomes, which have been separated by centrifugation (159). Sarcosomes do not respire in this case, but, when added to mitochondria, cause an increased O_2 consumption of the latter. In a homogenate, the fibrils remain relaxed as long as respiration persists.

Particulate matter from heart muscle has been studied morphologically and with respect to its dependence on the tonicity of the medium (160 to 164). Here too, they are the carriers of the oxidative system, and as such (or in a broken down form, the Keilin-Hartree oxidase preparation) continue to be widely used for studies on oxidation.

The particles in the flight muscles of insects are very numerous and large, 2.5μ in diameter (165); adenosinetriphosphatase and some oxidative enzymes have been demonstrated to be present (166, 167, 168).

In the heart, rhodanese is not present in the cytochondria although in liver it is a particulate enzyme (169); histochemical observations deal with enzymes in the intercalated discs of heart muscle fibers (170).

Structure of the myofibril.—Preparative isolation of myofibrils has been achieved after digestion of the tissue with trypsin or collagenase, or without such treatment (171 to 174). The newest method of Perry (173) is recommended (240).

Microscopic observations deal with the cross-striations in contracting glycerol-extracted fibers (175) and in isolated fibrils (176). Several further descriptions of structures visible by electron microscopy, and their changes in contraction, have appeared (157, 171, 171a, 184, 185, 328). Especially important are observations by Huxley & Hanson (177, 178); by differential extraction of myosin, they found this protein to be located in the A-bands. Cross sections through the A-band showed thinner and thicker filaments, the former extending throughout the I band as well, while the latter but not the former were present in the H bands. Most probably, the thicker filaments consist of myosin, the thinner ones of actin. The thicker filaments are about 110 \AA in diameter and are arranged in a perfect hexagonal pattern; in the H-

band, they are somewhat thicker and may be of different composition. A hexagonal periodicity of 440 Å, with interesting additional data, were also obtained by Huxley (179) by low-angle x-ray diffraction studies of living muscles.

The Lotmar-Picken diffraction diagram has been discussed in terms of its support to the helix theory of the structure of myosin (180, 181). However, two groups of investigators (182, 183) who have finally been able to reproduce this diagram, have ascribed it to a water-soluble constituent entirely unrelated to myosin, which occasionally crystallizes within the muscle structure.

An important investigation by Ströbel (186) in Weber's laboratory deals with the birefringence of contracting glycerol-extracted muscle fibers in terms of intrinsic and form-birefringence. The conclusion emerges that in contraction there is no disorientation of filaments, but an intramolecular or micellar contraction, presumably a folding of peptide chains.

The structure proteins.—The terminology in this field is obscured by the fact that the word "myosin," once used collectively for myosin and actomyosin, now applies to a single protein. We shall continue this usage, noting, however, that Weber (331, 332, 333) uses "myosin" in the collective sense, and refers to the pure substance as L-myosin. Dubuisson's (see 191) terminology remains in use to denote the electrophoretic components α , β and γ , corresponding to actomyosin, myosin, and contractin, respectively.

A procedure has been described for determining actin and myosin in muscle by differential extraction (187). Kjeldahl determination of muscle proteins should be done with care; erratic high results obtained for the refractive index increment (233, 254) indicate incomplete digestion. Further work has been done on the electrophoretic analysis of the myosins (191), e.g. of frog (188) and lobster muscle (189); the α and β myosins of smooth muscle, and of embryonic muscles, differ from those of adult striated muscle in their mobilities (190). Additional evidence has been recorded that the extractibility of the myosins is not merely determined by ATP (192); when the onset of rigor is delayed, the extractibility remains normal after all ATP has disappeared; likewise after freezing and thawing. Dubuisson (193) has demonstrated that the myosins become insoluble not only after prolonged activity, but also during one single contraction of turtle muscle; no contractin appears in this case. The cardiac proteins have been studied by electrophoresis (195, 195a, 196), as have glycogen-myosin complexes (196a).

Tropomyosin has been further studied by Bailey *et al.* It has a molecular weight of 53,000 in salt solution and polymerizes in the absence of salt (197, 198). Myosin and tropomyosin have no free N-terminal amino groups and are therefore considered as cyclical peptides (199). Amino acid analyses have been performed on myosin, actin, and tropomyosin; there is considerable similarity; the hypothesis has been proposed that myosin is a compound of the two other proteins (200). Isolated fibrils contain some water-soluble protein, one-third of which is tropomyosin; this constitutes 4 per cent of the to-

tal myofibrillar protein, corresponding to the total tropomyosin in muscle (201). With strong salt solution, actomyosin is extracted and no selective extraction of myosin is obtained (201). Fish muscle contains two proteins with solubility characteristics like tropomyosin; one is this protein itself, the other a compound with ribonucleic acid; the two proteins have been crystallized (202). The nucleotropomyosin contains up to 39 per cent nucleic acid; it is suggested that in muscle all the ribonucleic acid and tropomyosin are combined (203). No nucleic acid was detected in mammalian fibrils (201). Still more complex systems, also involving actomyosin, are thought to occur in smooth muscle (204). Myosin and actin show a slower turnover than average muscle protein (205); this, however, is not well established for actin, which was not purified.

Actin.—This protein has been purified by ultracentrifugal isolation of its polymer, followed by depolymerization at pH 8.2 in the presence of ATP (206); the product is pure in that it polymerizes quantitatively. Other methods of preparation have been proposed (209, 210), but the purity achieved has not been tested with the above criterion; the latter method is recommended as an improvement on Straub's original procedure to prepare crude actin, preceding ultracentrifugal purification. The protein has a molecular weight of 55,000 to 60,000, according to light-scattering measurements (206). The G-F transformation is ascribed to the following mechanism: salts abolish the repulsions between negatively charged proteins; once the molecules can collide, they combine to a linear polymer (207). [The acidification which occurs is not related to the polymerization process (207, 253)]. Light-scattering data and other physical properties are in support of this view, without providing exhaustive proof (207, 233). An alternative explanation was brought forward by Tsao (211), based on measurements of the depolarization of fluorescent light, emitted by actin coupled with 1-dimethylaminonaphthalene-5-sulfonylchloride. The molecular weight for the monomer was found as 75,000, in excellent agreement with the above value since this is essentially a determination of the rotary diffusion constant which is lowered by deviations from the spherical shape. F-actin, however, behaved as a dimer, and recourse had to be taken to long-range type interactions to explain the physical appearance of actin. The main uncertainty in this argument is that the decay time of fluorescence, which may change, was not measured.

Work with impure actin has permitted no certain conclusions regarding the participation of ATP; the best studies demonstrate dephosphorylation and deamination of nucleotides and other reactions (212, 212a, 212b). With pure actin, a simple stoichiometric equation has been obtained (208):



The amount of ATP dephosphorylated in this reaction corresponds to the extent of ATP breakdown in a fully activated muscle twitch (208, 213, 214), suggesting a physiological significance. The ADP formed is bound by F-actin, a combination which may be related to the bound ADP found in myofibrils

by Perry (173). The amount present agrees with this assumption, if allowance be made for some loss during the isolation of the fibrils.

Snellman & Gelotte (215) believe that the natural prosthetic group is different from ATP, and Gelotte (216) recently reported on a substance prepared from actin, with rather unprecedented properties. It has also been suggested that the phosphocreatine system is an essential cofactor (217), but this cannot be accepted since pure actin does not require this. Cortisone promotes actin polymerization (218).

Myosin.—Experience seems to indicate that the method of preparation developed in Weber's laboratory (331, 332) is generally preferable to that of Mommaerts & Parrish; a combination of the former with the crystallization according to Szent-Györgyi has been described (240). Myosin preparations from some mammalian species have been found indistinguishable in the ultracentrifuge (219). The binding of ions has been studied and subjected to careful theoretical analysis (220, 221).

An important development, initiated originally by Gergely (222, 223) working on adenosinetriphosphatase, is the fragmentation of myosin with trypsin. This has been investigated by Mihalyi & Szent-Györgyi (224 to 227). The splitting reaction leads to well defined products, L- and H-meromyosin. The former resembles myosin in its solubility characteristics and can be crystallized; the latter is soluble at low salt concentrations, but carries the adenosinetriphosphatase and actin-combining activities (226). The total adenosinetriphosphatase undergoes very little change in the process (222, 223, 226). The heavy component has a $S_{20,w}$ at zero concentration of 6.96×10^{-12} and a diffusion constant of 2.9×10^{-7} ; for the light component, these values are 2.86 and 2.87 respectively. Preliminary values for the molecular weights are 232,000 and 96,000 (227). The sedimentation constants give the impression that H-meromyosin must have folded or coiled when liberated. While myosin has no free end groups (199), preliminary data indicate that lysine and alanine are the N-terminal amino acids in H-, and aspartic acid and glycine in L-meromyosin (227). All the free SH-groups of myosin are in the heavy moiety (227). Fragmentation of myosin with urea, guanidine, acid, or alkali (229) has not given as clear results as the work with trypsin; it brought forth no evidence that tropomyosin is a part of the myosin molecule; nor could adenosinetriphosphatase be detached by these methods.

The problem of the molecular dimensions of myosin, seemingly solved in Weber's laboratory (331, 332), is in reality more complicated. Parrish & Mommaerts (230) find that $S_{20,w}$ has no singular value, but depends on the temperature. Scattered data in the literature (219, 231) also contain indications in that direction. A marked boundary-sharpening effect occurs (230). While orientating light-scattering measurements indicated a molecular weight around 10^6 (232, 233), recent extensive work (228) suggests a value of only 700,000, obtained both by scattering, and transmission measurements. The molecular length is 1600 Å (228).

Adenosinetriphosphatase.—The electrochemical properties of ATP and

its hydrolysis products are such that, in a broad pH range, acid is produced in its splitting (11). An accurate electrotitrimetric method for the measurement of adenosinetriphosphatase has been developed on that basis (234), and a simplified modification is useful as a screening-test (235).

Sarcosomes contain a magnesium-activated adenosinetriphosphatase, apparently identical with the Kielley-Meyerhof enzyme (155, 157, 158). A similar adenosinetriphosphatase also occurs in insect muscle mitochondria (167, 236). This is inhibited by mercurials and, competitively, by ADP, although ADP is slowly split (236). Insect muscle contains myokinase (167) and an apyrase which shows inhibition by excess substrate at low, but not at higher, Mg concentration (237). Fibrillar adenosinetriphosphatase, on the other hand, is always of the myosin type and is calcium activated (167, 172, 238).

It has been known for years that myosin-adenosinetriphosphatase requires adenylate kinase to remove the second phosphate of ATP; the complete removal of this is cumbersome (239) and seems to be promoted by precipitation with ATP (240). By use of ATP even myofibrils can be depleted of adenylate kinase, which is otherwise not feasible (172). A convenient preparation of adenosinetriphosphatase for analytical purposes has been described (241). When adenylate kinase is suppressed, direct deamination of ADP occurs in myofibrils of skeletal muscle (242). Inhibition of myosin adenosinetriphosphatase by oxidants (243) and colchicin (244) has been studied.

Reinvestigating pure myosin in entirely fresh condition, Mommaerts & Green (245) find higher activities and more profound ion effects than had been known before. At pH 6.3 with Ca the enzyme might almost account for the *in vivo* requirements, but Mg inhibition is pronounced. Hasselbach (246) states that actomyosin is activated by Mg, but this is found at pH 7, where the maximal activity is not much higher than the inhibited one at the pH optimum. We still doubt, therefore, that myosin-adenosinetriphosphatase is sufficient to explain activity metabolism (334).

A beginning has been made with kinetic studies on myosin-adenosinetriphosphatase. Gergely (248) determines $K_s = 3.5 \times 10^{-4}$ for the ITP splitting by H-meromyosin and finds $K_s < 10^{-4}$ for ATP. Ouellet *et al.* (249) and Watanabe *et al.* (250, 264) report values of K_s for ATP. The former, using analytical methods that may not be adequate, find that at pH 7, inhibition by Mg does not change K_s , and they conclude that $K_s = k_2/k_1$; the latter, however, do find a variation of K_s . Mommaerts & Green (251) find that at pH 6.3, K_s varies strongly when k_2 changes, and is, therefore, definitely not an equilibrium constant. Laidler *et al.* (252a) draw far-reaching conclusions from the effect of methanol on adenosinetriphosphatase, which are premature in view of the uncertainty concerning K_s . Myosin is inhibited competitively by ADP (251). At high ATP concentrations, substrate inhibition can occur (246, 247, 250).

There is evidence that myosin and adenylate deaminase can be separated (240, 252).

Actomyosin.—The effect of ATP upon actomyosin in 0.5 M KCl is gen-

erally accepted to consist of a dissociation of the protein into its components (260) although, in the ultracentrifuge, actin is not seen. Blum & Morales (254), however, conclude that ATP causes an extension of actomyosin particles; this theory was based on light-scattering measurements with the extrapolation method, but the technique (wide photocell angle, wide beam; measurements to 45° only, requiring uncertain extrapolation) was not sufficient to establish the point with certainty. At alkaline reaction, dissociation was found (255) in agreement with the conventional view. Other difficulties were raised by Tsao (256); his work, however, is subject to the uncertainty alluded to when discussed previously (see p. 390). Actomyosin has been found to dissociate without ATP at low temperatures (257, 264), indicating that the association is endothermic (257). The formation of the complex causes no pH change (258). Earlier work on SH-groups in actomyosin and its constituents has been continued (259). The viscosity of actomyosin and its components has been further investigated (260, 261). Insect actomyosins have been studied (238).

Earlier viscosimetric work on the effect of ATP upon actomyosin did not permit kinetic observations on the first stages of the reactions. Such studies have now begun, using rapid recording viscosimetry (262, 263), or light-scattering (264, 265). The former authors found the rate of combination to be little dependent on pH, proportional to [ATP], accelerated by Mg, and inhibited by Ca at low concentrations and stimulated at high concentrations; the latter workers obtained similar results and aim at a connection with the kinetics of adenosinetriphosphatase. It is estimated that the equivalent weight of myosin, reacting with one mole of ATP, is about 140,000 (264), in agreement with earlier estimates (334).

Various aspects of the effect of ATP upon actomyosin gels and suspensions have been described in great detail (266 to 271). The splitting of ATP in these reactions has been established (272). The splitting of ATP is concluded not to be directly involved in the contraction of actomyosin threads, because of contrary effects of Ca and Mg upon the two processes (273, 274, 275).

Contraction and relaxation.—Although the actomyosin threads mentioned in the previous section contract, they are imperfect models through their deficient molecular orientation and cross-linkage. Threads which contract unidirectionally and can develop tension or lift a weight, have been prepared in two ways. Hayashi (276, 277) prepares such threads from surface-spread actomyosin, by lateral compression of the layers. Portzehl (278, 279) achieves the same by carefully controlled drying of conventional actomyosin threads. Such preparations display several characteristic properties of muscle. While the psoas fiber (*vide infra*) may be a superior object, these artificial fibers form an important link with simpler protein systems (280).

Using Hayashi's technique, Kafani & Engelhardt (281) made the important discovery that myosin itself, without actin, can form a contractile system with ATP. The conditions under which these fibers shorten are different

than for actomyosin; the optimal pH is 9, but the tension and shortening can be equal. Whether the myosin contained traces of actin is an academic question only, since it was previously believed that at least 6 per cent actin was needed to make a contractile system (282). It is not denied, of course, that actin plays a role in muscle, to establish the proper conditions for myosin. Contractility of myosin, in the superprecipitation test, could further be established at nearly neutral reaction after combination with certain dyes (283).

Weber *et al.* (332, 333) have developed further the Szent-Györgyi fiber preparation (335), obtained by extracting fiber bundles from the rabbit psoas muscle with aqueous glycerol. Fibers of this nature have also been prepared from cardiac (289) and smooth muscles (285, 288). While the variation of tension with temperature in this preparation had been ascribed previously to an endothermic equilibrium between contracted and relaxed elements, it is now clear that no such equilibrium prevails, but that there is a stationary state (reproducibly determined by the temperature) between ATP diffusion into the fiber and its enzymatic breakdown; conceivably, inhibition by ADP may be an additional parameter. The diffusion constant of ATP in the fiber has been determined from the limiting fiber thickness which permits maximal ATP splitting or full contraction under certain assumptions [$D' = 2 \times 10^{-8}$, 100 times smaller than in free diffusion (284)]; this permits experimentation with fibers of 30 μ thick [60 μ for smooth muscle (285), or actomyosin threads (279)]. With these fibers and actomyosin threads, Weber & Portzehl have studied a number of mechanical properties, such as the "quick release" phenomenon, and elastic modulus (286, 290). Freshly prepared fibers are rigid, resembling rigor muscle. Some substances, "plasticizers," make them extensible; the best plasticizer is ATP, but its softening effect is concealed by the contraction, unless the latter is inhibited by mercurials [see (299) for some related data]. These results are extensively discussed in two important summaries (332, 333). With regard to contraction, Weber & Portzehl present the following view: combination of ATP with actomyosin plasticizes the system to permit molecular mobility; splitting of ATP causes contraction. The main argument in favor of this view is that mercurials, such as mersalyl (Salyrgan) inhibit both contraction and adenosinetriphosphatase (although this may have other explanations, as the authors themselves point out); the effect of the relaxation factor (*vide infra*) which inhibits adenosinetriphosphatase (287, 298) is also in line with this, as is a parallelism in the effects of ATP concentration upon splitting and contraction (288a). The effects of ions, particularly Ca which activates adenosinetriphosphatase yet inhibits contraction, give less unequivocal support, or even arguments to the contrary (273, 274, 275). More work will be required in this direction, unless a substance were found which blocks adenosinetriphosphatase without inhibiting contraction, which would directly prove the opposite view.

Bozler's investigations (291 to 294) on the psoas fiber differ from the previous work in that his preparations are only briefly extracted and so contain additional factors. By changes in ionic composition, the contraction cycle can

be reproduced *in vitro* but, while this is a significant demonstration, it does not allow a clear analysis as yet since the effects of, e.g., Mg or Ca upon the contraction and relaxation factors overlap. Simultaneous investigation of contraction and ATP-splitting by such fibers gave interesting results. In the relaxed state, splitting was low; upon contraction with Ca, it rose five-fold (295). It was further established (296) that in the presence of phosphocreatine (see also 283), both contraction and relaxation are caused efficiently by small amounts of ATP. About $0.4 \mu\text{M}$ nucleotide is required per gm. muscle, which is the same as is involved in one muscle twitch (208, 213, 214, 334).

The most important advance in the understanding of relaxation is the discovery of a relaxation factor. Marsh (297) described a syneresis of homogenized muscle, apparently attributable to contraction of the disintegrated fibers and its reversal by a water-soluble protein and ATP. Bendall (298) studied the effect of this factor upon the glycerol-extracted fiber bundle. This preparation, after contraction in 0.16 M KCl , 0.004 M MgCl_2 , and 0.006 M ATP , would reversibly relax in the presence of a soluble muscle protein in the same medium; Ca was a potent inhibitor for this process and reactivated contraction. These results go far in explaining many of Bozler's findings (*vide supra*). The Marsh-Bendall factor also inhibits the adenosinetriphosphatase activity of the fibers (297, 298) and of actomyosin (287) and effects in re-establishing the extractibility of myosin from aged minced muscle (194).

The contraction cycle has been reproduced in a different manner by Goodall & Szent-Györgyi (300). In the presence of ATP, phosphocreatine, Mg, and water-soluble proteins, contraction occurs at pH 7.5, relaxation at pH 6.0. Without the additional protein, ATP and phosphocreatine cause only contraction. Lorand (301), in Szent-Györgyi's laboratory, has elaborated on these results. Evidence was obtained that the relaxing effect is related to a rephosphorylation of ADP, and pure creatine phosphokinase was found to cause relaxation, if incompletely. The relation between these experiments and Bendall's is hard to evaluate, since the MB-factor acts without phosphocreatine or other dialyzable $\sim\text{P}$ -donors; instead, adenylate kinase might be involved (301), but this is probably already contained in the fiber (cf. 240); the regulation of pH in the one, and its constancy in the other group of experiments, makes a comparison difficult. ATP can also cause the contraction of living muscle (e.g., 302), but this has now been shown to be an indirect effect, through the acetylcholine mechanism (303, 304).

The physiology of living muscle is beyond the scope of this review, but reference must be made to several papers by A.V. Hill (305, 306, 307), as well as some reviews (308 to 311). D.K. Hill (312, 313) has continued his studies on optical changes in contraction.

Theory of contraction.—The "window field" theory (326, 326a) attempts to make a connection between excitation and activation of the contractile matter. Two theories have been developed (314 to 317, and 318) with the aim to explain contraction and other properties in terms of the transition be-

tween long and short molecular units; these theories have important features, but cannot be related briefly. Other types of theories (319 to 323) originate from comparisons with the thermokinetic contraction of rubber, first proposed by Wöhlisch in 1941. A recent formulation (321), however, may open the door to bringing some of such work in line with other views. Theories closely adhering to the rubber-like elasticity model have now been definitely disproved by experimental application of its own main criterion: Hill [(324), see also Aubert (325)] has demonstrated that "when the tension of a muscle contracting isometrically is rapidly lowered, there is an immediate and proportional rise of temperature," and concludes that "during active contraction, therefore, the contractile filaments possess normal and not long-range elasticity. The force exerted by active muscle is not of thermokinetic origin." Further theoretical developments are first of all dependent on the acquisition of fundamental facts, too few of which are yet available.

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BIOCHEMISTRY OF HORMONES (RESTRICTED TO PITUITARY AND ADRENAL INTERRELATIONSHIP)^{1,2}

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INTRODUCTION

Nonsteroid hormones were not reviewed as such in Volume 22 (1953) of the *Annual Review of Biochemistry* and, although references were made to ACTH in the article on "Chemistry of Amino Acids, Peptides, and Proteins" (e.g., p. 659), the subject of ACTH (Corticotropin) has not been reviewed comprehensively in these volumes since the article by Li & Harris (1). Since the amount of research carried out on ACTH during the past two years has been substantial, the present review will be devoted largely to a consideration of this, though recent developments in research on the nature of the adrenal cortical secretions will also be touched upon.

Of the many reviews relevant to the subject of the present article which have been published during the past two years, the following will be quoted: Astwood, Raben & Payne (2), Ballabio, Cavallero & Sala (3), Bergstrand (4), Ingle (5), Ingle & Baker (6), Klyne, Wolstenholme & Cameron (7), Li (8 to 11), Miner (12), Pirani (13), Ralli (14), Thorn *et al.* in a series of articles published during February, March, and April, 1953 entitled "Pharmacological Aspects of Adrenocortical Steroids and ACTH in Man," which include nearly 700 references up to the end of 1952 (15), Waring & Ketterer (16), White (17), Yoffey (18), and Young (19).

DEFINITION OF ACTH

Many different types of biological assessment of ACTH (Corticotropin) activity have been used during the past few years; but the original concept of ACTH was that of a substance present in, and presumably secreted by, the anterior lobe of the hypophysis which, on parenteral administration, was capable of maintaining or restoring the weight and function of the adrenal cortices of the hypophysectomized rat. The first preparations of the "protein-hormone" certainly possessed these biological properties. With the development by Sayers and his colleagues of rapid micromethods for the assay of ACTH, based on the rapid fall of adrenal ascorbic acid in hypophysectomized rats following a single intravenous injection of the solution under test, the "maintenance" or "repair" assays for ACTH, both time-consuming and prodigal of material, fell largely into disuse. The subsequent introduction of

¹ The survey of the literature pertaining to this review was completed in September, 1953.

² The following abbreviations are used in this review: A. A. for ascorbic acid-reducing; A. W. for adrenal weight-increasing.

assay methods based on a fall in the blood lymphocyte or eosinophil count still further complicated the picture, particularly since the results with one method of assay could often not be correlated with those of another. Some methods which are commonly in use for the detection and assay of ACTH include (a) various forms of the "maintenance" and "repair" test, (b) the Sayers test (by which is meant a test or assay based on the fall of the adrenal ascorbic acid in hypophysectomized rats which follows intravenously administered ACTH), (c) the reduction of thymus weight in the nestling intact rat when ACTH is administered subcutaneously in a medium designed to delay absorption, for example mixed with beeswax and arachis oil or precipitated with alum (20 to 23), (d) the fall of blood eosinophils in intact or hypophysectomized mice (24 to 27), (e) the increase in urinary excretion of 17-hydroxycorticosteroids in man (26, 27) or guinea pig (28), and (f) the *in vitro* stimulation of the metabolism of isolated adrenal preparations or slices, with a rise in oxygen consumption (29 to 32) or an increase in adrenal steroid production, as a criterion of ACTH activity (33 to 37). Variations in the standard Sayers test have been based mainly on subcutaneously administered ACTH, as opposed to the normal intravenous route (38, 39), though the inhibiting effect of treatment with cortisone acetate on stress-induced depletion of adrenal ascorbic acid content in intact rats has been utilized to avoid the need for hypophysectomized rats in the assay of ACTH by the Sayers assay (40). The influence of ACTH on the liver weight of normal guinea pigs and rabbits (41) is also a possible basis for the assay of ACTH. With such a multiplicity of methods available it is not surprising that quantitative discrepancies are frequently found between the results of different methods. Moreover, quantitatively different results are obtained with some preparations depending on whether an intravenous or subcutaneous route of administration is adopted (23, 38, 39, 42, 43). The possible significance of these findings is discussed later.

Claims have been made that certain sex hormones are able to exert an ACTH-like action, but a former claim that treatment with oestrogen can synergize the action of pituitary implants on the adrenal of the hypophysectomized rat has now been withdrawn (44). Some ACTH-like activity has been claimed both for testosterone (45) and for methylandrostenediol, a substance weakly androgenic but active in inducing protein deposition in tissues (46); testosterone prevents the adrenal atrophy which normally follows treatment with cortisone in intact rats (47), though methylandrostenediol is even more effective in this respect in the rat (48, 49, 50). Nevertheless, since methylandrostenediol is without an action of this sort in the cortisone-treated rhesus monkey (51) species differences may complicate this finding. Winter *et al.* (48) find that treatment with methylandrostenediol plus cortisone largely repairs the atrophied adrenals of hypophysectomized rats. This interesting observation at present lacks independent confirmation, but its possible implication that variations in the proportion of androgen in the total

adrenal steroid secretion induced by ACTH might account for the different relative effects of various preparations of ACTH in maintenance or repair tests on the one hand and in ascorbic acid depletion tests on the other, is one to be considered.

The preparation from placental tissue of small amounts of material active in the Sayers test for ACTH has been both affirmed (52 to 55) and denied (56), and although it has been claimed that purified preparations of the active substance resemble ACTH in chemical and physical properties (55), it is unwise to presume that the substance (or substances) present in placenta is indeed ACTH, particularly when it is remembered that steroid hormones of various type are found in placenta. Moreover, since in the hypophysectomized rat prolonged stress (for example a large burn) (57) or treatment with epinephrine (adrenaline) (58), can induce a histological appearance of the adrenal glands consonant with stimulation, the need for caution in evaluating the results of experiments concerned with the possible ACTH-like activity of miscellaneous substances is clear.

THE SIGNIFICANCE OF ACTH "PROTEIN-HORMONE"

The view that ACTH is a protein of molecular weight about 20,000 was troubled by many earlier claims that ACTH activity is dialyzable. More recently Astwood, Raben & Payne (2) treated "protein-hormone" dissolved in 0.1 *N* acetic acid with either cellulose or oxycellulose and recovered the major part of the biological activity in the material eluted from the adsorbent by 0.1 *N* HCl, while the unadsorbed fraction was unaltered electrophoretically despite the fact that most of the active material had been removed. Similar results were obtained by Dixon *et al.* (59) with ion exchange resins, and the conclusion seemed probable that the hormone was in fact a minor contaminant of the so-called protein-hormone from which it could be released by dialysis, adsorption, or ion exchange. In a reexamination of the properties of "protein-hormone" Li & Pedersen (60) reaffirm that the protein prepared from sheep pituitary glands has a molecular weight of 20,000 and is essentially homogeneous in the ultracentrifuge. In acid solution (0.05 *N* HCl+0.05 *N* acetic acid or 2 *N* acetic acid) dissociation of protein into smaller particles was demonstrated both by dialysis and by studies with the ultracentrifuge (60). In the acetic acid solution 20 to 40 per cent of the protein became dialyzable, while the nondialyzable fraction possessed the same sedimentation constant as the original protein. The dialyzable and nondialyzable fractions possessed similar biological activities in the Sayers test, and the authors suggest the possible existence of more than one active center on the protein hormone (60). Mendenhall (61) has recently published an amino acid analysis of ACTH protein hormone though data for tryptophan are lacking; the least molecular weight calculated thus is approximately 23,000, the number of amino acid residues in the molecule being 185 to 190. The cystine content is 8.0 per cent while leucine and isoleucine are present to the extent of 7.5 and

3.2 per cent respectively; 3.0 per cent of threonine is also found. It is of interest that these four amino acids are absent from some active ACTH preparations (see later).

Li and his colleagues have subjected sheep protein-hormone to counter-current distribution (62) and find that much of the biological activity is concentrated in small fractions by weight. Thus with counter-current distribution with the system 2,4,6 collidine water (the 2,4,6 trimethyl isomer) at least three components are revealed in otherwise apparently homogenous sheep-protein hormone (62). The main component comprised 61 per cent of the dry weight and was practically devoid of potency in the Sayers test, while a minor component representing 16 per cent of the dry weight possessed most of the biological activity. McDonald & Marbach (63) subjected pig protein hormone to paper electrophoresis at pH 5.5 in veronal buffer and obtained a cathodic fraction containing 31 per cent of the total N and possessing about 98 per cent of the total activity in the Sayers test. The mobility of this fraction at pH 6.0 to 6.6 approached zero. In discussing the significance of the protein-hormone Astwood, Raben & Payne (2) point out that since a substance 100 to 150 times as active as the protein hormone has been prepared whose molecular weight is estimated to lie between 2,500 and 10,000 it is manifestly impossible that such a substance could be a fragment of a native hormone protein one-hundredth as potent and of molecular weight 20,000. In the opinion of the present reviewers, evidence is lacking that the protein-hormone has either chemical or biological significance.

THE PROPERTIES OF MATERIAL ACTIVE IN THE SAYERS TEST

The observation by Li that biological activity survived treatment of protein-hormone ACTH with pepsin or acid under conditions in which incomplete hydrolysis of the protein might be expected, was at first interpreted as involving the release by hydrolysis of a small and biologically active peptide which constituted a structural fragment of the protein-hormone. There appears to be no good evidence to support this view. Nevertheless, hydrolysis by pepsin or acid in the preparation of ACTH has been employed by many groups of investigators, and the methods of preparation employed and the properties of the concentrates so obtained, will first be considered.

Preparations obtained by a method involving an obvious hydrolytic step.—In a series of papers from the Research Laboratories of Merck & Co. (64 to 68) the preparations of "corticotropin B" from pig pituitaries is described, this being so designated because its properties differ from those of (unhydrolyzed) corticotropin (65). The procedure involved extraction of acetone-defatted pig glands with methanolic acetic acid, the active material then being adsorbed on oxycellulose according to the method of Astwood *et al.* (2). Elution was followed by digestion with pepsin at pH 2.5 for 24 hr. at 37°C., and precipitation of impurities by 5 per cent trichloroacetic acid. The corticotropin B concentrate was further purified by counter-current distribution in the system *S*-butyl alcohol/0.5 per cent aqueous trichloroacetic acid. The acetate

salt of the corticotropin B was then isolated with the aid of the ion-exchange resin IRA-400 (68). Alternatively the corticotropin B concentrate was purified by exchange with the ion-exchange resin Amberlite IRC-50 (67). The presence of two components other than corticotropin B in concentrates purified by ion-exchange procedures was revealed by counter-current distribution in the system *S*-butyl alcohol/0.5 per cent aqueous trichloroacetic acid involving 200 to 450 transfers. Comparison of the observed and calculated curves indicated an approximate purity of 95 per cent. The best preparations possessed an activity of 300 international units/mg.

White & Fierce (69) have utilized the cation exchange resin Amberlite XE-97³ for the fractionation of acid and pepsin-hydrolyzed pig ACTH, and conclude that two types (in their terminology types II and III) of active fraction can be differentiated by performance on the resin column and can thus be separated from unhydrolyzed ACTH (type I). The activities of the type II and type III preparations obtained were 100 to 150 I.U./mg. When an oxycellulose eluate (type I) was treated with pepsin the activity was solely of type II within 2 hr. but even after 24 hr. no appreciable amount of type III had been formed. Nevertheless, subsequent treatment with acid converted at least a part of type II into type III. The authors suggest (69) that type II activity must have predominated in the ACTH concentrate from which corticotropin B was isolated by Brink *et al.* (65 to 68).

Li and his colleagues studied the fractionation of pepsin hydrolysates of ACTH from sheep (10, 70, 71, 72) and pig (71) glands by means of fractionation with trichloroacetic acid (70), by counter-current distribution in the system collidine/water (71), and by the method developed by Tiselius & Hagdahl (73) involving carrier displacement chromatography (70, 72). No qualitative differences between hydrolysates of pig and sheep preparations were observed when these concentrates were distributed in the system collidine/water (71). By this means preparations of 10 to 20 I.U./mg. were obtained, although activity was shared among a number of different fractions. Smith *et al.* (74) treated acid aqueous acetone extracts of pig or ox pituitary glands with lithium chloride at pH 5.4; under these conditions inert material precipitated and active material was then separately precipitated by the addition of excess acetone. Exposure of the concentrates to 100°C. in 1.0 *N* HCl for 30 min. destroyed growth hormone, gonadotropins, thyrotropin, and posterior pituitary antidiuretic hormone, and yielded material of 10 to 20 I.U./mg.

In the experiments of Loomer & Witter (75) paper electrophoresis of a commercial ACTH preparation yielded at least four active components, though the 5 per cent trichloroacetic acid-soluble portion of the starting material was electrophoretically homogeneous and contained 80 per cent of the biological activity and 6 per cent of the dry weight. The biological activity of the material is hard to evaluate from the data given in this paper.

³ Amberlite XE-97 closely resembles Amberlite IRC-50 and Amberlite XE-64.

Li (10, 76) has repeatedly claimed that an increase in activity can be obtained by partial acid hydrolysis of ACTH. Thus when a 1 per cent solution of an oxycellulose concentrate in 0.2 *N* HCl was kept at 100°C. for 3 hr. the potency was increased approximately six-fold (76). So far the claim to have obtained an increase in activity by partial acid hydrolysis lacks confirmation and must at present be regarded as unproven.

Preparations obtained by a method involving no obvious hydrolysis.—The substance active in the Sayers test and representative of the unhydrolyzed pituitary preparations, has been variously designated corticotropin (66), "corticotropin purified" (42), "corticotropin A" (77), and "corticotropin type I" (69). Moreover, there are important differences between unhydrolyzed ACTH before and after it has been subjected to adsorption on and elution from oxycellulose. Consequently Wolfson (42) distinguishes between "Corticotropin crude" (the familiar ACTH-corticotropin, which has been generally available at an activity of about 1 I.U./mg.), "Corticotropin purified" (also described by him as "high potency corticotropin," represented by oxycellulose eluate and designated "ACTX-corticotropin" by him), and finally the highly purified material isolated from peptic hydrolysates of crude corticotropin (which Wolfson calls "ACTIDE-corticotropin"). In the present review the term "Corticotropin A" will be used to describe the active material concentrated from unhydrolyzed preparations of ACTH by a process involving adsorption on and elution from oxycellulose or ion-exchange resins, while "corticotropin B" will be used to designate material concentrated from pepsin hydrolysates of crude ACTH. Our "corticotropin B," that of Brink *et al.* (65, 66, 67), "corticotropin type II" of White & Fierce (69) and "ACTIDE-corticotropin" of Wolfson (42) are similar, while the "corticotropin" of Brink *et al.* (65, 66), the "corticotropin A" of White (77), the "corticotropin type I" of White & Fierce (69), and the "ACTX-corticotropin" of Wolfson, are all similar, and will be discussed under the designation "corticotropin A" in this review.

Dixon *et al.* (59) were the first to apply ion-exchange methods to purification of unhydrolyzed ACTH and obtained two main and a number of subsidiary fractions by this means (59, 78, 79, 80), the maximum activity observed being not more than 100 I.U./mg. These authors consider the possibility that there exists more than one "corticotropin A." Li and his colleagues (10, 62, 76, 81, 82) have subjected hydrolyzed sheep pituitary preparations to oxycellulose adsorption and elution, followed by partition chromatography on a column of Kieselguhr with 0.2 *N* HCl saturated with isobutyric acid as the stationary phase, and a moving phase of isobutyric acid saturated with 0.2 *N* HCl. The most active fraction contained 10 per cent of the total N and 95 per cent of the activity, and ran consistently on rechromatography to yield a symmetrical peak. The authors conclude that this fraction, which exhibited a biological activity of 200 to 400 I.U./mg., showed no gross inhomogeneity by the criterion of partition chromatography which was applied. Dedman *et al.* (83, 84) have continued to emphasize the

ultrafilterability of ACTH, a property which is somewhat neglected by other workers. Like Astwood (2), Dedman *et al.* (83, 84) have utilized acetic acid for the extraction of defatted and desiccated pituitary tissue (pig) and find that the biological activity is extracted almost quantitatively by 99.5 per cent acetic acid; active material can be precipitated from this extract by the addition of ether. They state (84) that the stability of the preparation is improved if the acetic acid extract is heated to 100°C. for 15 min. before ether precipitation and that although biological activity is completely unchanged by this process, the chemical properties are somewhat altered. When the extract had not been thus heated, a solution of the ether precipitate in 0.2 *N* formic acid could be ultrafiltered at 40°C., under which conditions 90 to 100 per cent of the biological activity passed through the membrane accompanied by 20 to 30 per cent of the weight. When, however, the acetic acid extract had been heated, only about 30 per cent of the biological activity was ultrafilterable in formic acid at 40°C. These authors suggest that in addition to destroying the proteolytic enzymes, heating in acetic acid brings about some change in the accompanying proteins which makes it more difficult to separate the smaller molecular ACTH from them (84). The present reviewers assume in the absence of precise evidence that the material thus obtained in not hydrolyzed in any appreciable degree, though some other change may have occurred, and that, therefore, the product is akin to corticotropin A rather than to corticotropin B. Dedman *et al.* (84) emphasize that the specific effect of acetic and formic acids in facilitating the separation of ACTH by ultrafiltration is probably to be explained by the dissociating effect of these acids on proteins. Under suitable conditions ultrafiltration followed by fractionation with trichloroacetic acid yielded a preparation assaying 20 I.U./mg.

In a paper entitled "The isolation of corticotropin A" White (77) has subjected the slow-moving fraction from Amberlite XE-97 columns [type I corticotropin of White & Fierce (69)] to a 200 plate counter-current distribution in the system *S*-butyl alcohol: 0.2 per cent trichloroacetic acid. The activity was found in a single peak, broader than theoretical, with a distribution coefficient of about 1.75. Material from the centre of this peak, falling well within the limits of the theoretical curve for $k = 1.75$ was recovered as the trichloroacetate and redistributed in the same system. A comparison of the actual and theoretical curves indicated a purity of about 93 per cent; the product possessed an activity of 100 to 150 I.U./mg. These authors emphasize that biological activity of this order has been obtained only when hydrogen sulfide has been used in the distribution system. In the absence of an antioxidant, considerable losses in activity have been suffered, without any substantial change in redistribution pattern..

The properties of purified materials.—Until recently most attention has been directed towards the purification of materials from pepsin-hydrolysates of crude ACTH, and the properties of corticotropin B will first be considered.

Corticotropin B: According to Brink *et al.* (85) Corticotropin B isolated

as the acetate salt (65 to 68) is a white amorphous solid which is soluble in water and in methanol, but insoluble in benzene, ether, and other similar solvents. The specimens examined were found to behave in countercurrent distribution as though they contained not more than 5 per cent of foreign material. The isolated substance contained no phosphorus and no carbohydrate; N=17.6 per cent; S=0.5 per cent. The authors believe that there can be no doubt of its gross polypeptide nature though they do not rule out the possible existence of an undetected prosthetic group. The infrared spectrum disclosed only the existence of groupings associated with polypeptides.

The amino acid composition is given in Table I, the values having been determined by the method of Moore & Stein (86) on an acid hydrolysate, with the exception of tryptophan which was determined separately by the method of Shaw & McFarlane (87). The authors state that the known basic character of corticotropin B is clearly reflected in the preponderance of the basic amino acids, although the isoelectric point is not specified. The authors infer from the molar ratios of the amino acids present, which they consider to be gratifyingly near to whole number ratios, that the corticotropin B molecule contains about 60 amino acid units, or an integral multiple thereof and has a minimum molecular weight of the order of 6,000 to 7,000. The minimum molecular weight calculated from its behaviour in the ultracentrifuge was 5,200.

Corticotropin B contained no leucine, isoleucine, threonine, or cystine, all of which are found in the protein-hormone ACTH (61). The absence of cystine is interesting in that almost all workers in the ACTH field have found it advisable to use antioxidants in the course of extraction and preparation; Brink *et al.* observed (68) that the biological activity of ACTH solutions could be greatly reduced by bubbling air through at pH 7 in the presence of ferric chloride, but that reactivation occurred if the solution was freed from metallic ions by treatment with Amberlite IR-100 and saturated with H₂S at pH 4. Such behaviour might be expected from a sulfhydryl-disulfide oxidation-reduction equilibrium, although no evidence of such reactivation of inactivated solutions was observed by White (77), who finds that the influence of the addition of H₂S is that of protection against loss of activity only.

In a recent report comparing the properties of corticotropin A and B, Hays & White (88) state that the N-terminus of both is serine and that the order of amino acids from the N-terminus is, in the terminology of Sanger (89), Ser.Tyr.Met.Glu. The methods utilized in this investigation are discussed below. Hays & White (88) also report that in collaboration with Dr. David F. Waugh they have obtained by means of the ultracentrifuge, a value of approximately 2,300 for the molecular weight of corticotropin B at low pH (0.05 N HCl). They point out that this value is approximately one-half that found by Brink *et al.* (85), presumably at a higher pH and might indicate that corticotropin B dissociates at low pH and exists as a dimer near neutrality.

Corticotropin A: Until recently comparatively little effort had been de-

TABLE I
THE AMINO ACID COMPOSITION OF CORTICOTROPIN B*

	Sample 1†		Sample 2§		Sample 3§	
	A†	B†	A†	B†	A†	B†
Glycine	6.4	6.0	6.9	6.5	6.9	5.9
Alanine	2.3	2.2	2.4	2.1	2.6	2.1
Valine	6.3	5.9	5.4	4.8	6.7	5.6
Leucine	none	—	—	—	—	—
Isoleucine	none	—	—	—	—	—
Proline	5.5	5.1	7.0	6.4	6.2	5.2
Serine	4.7	4.3	3.1	2.9	3.1	2.6
Threonine	none	—	—	—	—	—
Phenylalanine	3.1	2.9	2.2	2.0	2.8	2.1
Tyrosine	4.2	3.9	4.4	4.0	4.9	4.1
Cystine	none	—	—	—	—	—
Methionine	1.9	1.7	1.8	1.6	1.6	1.5
Aspartic acid	4.4	4.1	4.8	4.3	4.7	4.0
Glutamic acid	6.0	5.7	6.0	5.4	6.1	5.1
Histidine	6.6	2.0	5.9	1.8	6.6	1.8
Lysine	17.0	8.0	16.7	7.6	16.0	6.9
Arginine	28.0	6.5	29.2	6.6	28.1	6.0
Ammonia	3.1	2.9	3.3	3.0	3.9	3.3
(Tryptophan		3.7)				
Total			98.8		100.2	

* After Brink *et al.* (85).

† A = per cent total N in acid hydrolysate

B = best calculated molar ratio

‡ Sample 1 prepared by countercurrent distribution.

§ Samples 2 and 3 purified to 300 I.U./mg. by treatment with oxycellulose (Sample 2) and Amberlite IRC 50 (Sample 3).

|| The contribution of the tryptophan nitrogen in the acid hydrolysate is included in the value for ammonia.

voted to the purification of unhydrolyzed preparations of ACTH, but within the past year, realization of the superior activity of the unhydrolyzed preparations over hydrolyzed materials, when given intramuscularly, has turned much attention to the chemistry of corticotropin A. Li (10, 76, 81, 90) believes that although ACTH has not been isolated in pure form, there is little doubt that the hormone is a polypeptide with an isoelectric point in the neighbourhood of pH 9 (10, 76, 90). He is of the opinion that the final purified material is likely to possess a potency higher than 400 I.U./mg. The following amino acids were found to be absent from the purest preparations obtained in his laboratory: cystine, methionine, serine, threonine, isoleucine,

and histidine (10). Reaction with ketene, nitrous acid, and acetic anhydride indicates that free amino-groups are essential for biological activity (10). In the best preparations 3 per cent of tryptophan and 5 per cent of tyrosine (cf. however, 10) were found (76).

White (77), Landmann, Drake & White (91), White (92), and Hays & White (88) have recorded the presence of the following amino acids in corticotropin A: arginine, histidine, lysine, phenylalanine, serine, leucine, valine, glutamic acid, tyrosine, aspartic acid, glycine, alanine, proline, tryptophan, methionine. Of these amino acids leucine is the only one not found in corticotropin B (85). From the available data concerning amino acid composition (77, 88) it appears that corticotropin A has the following empirical formula: Ala.₂ Arg.₂ Asp.₁ Glu.₄ Gly.₂ His.₁ Leu.₃ Lys.₃ Met.₁ Phe.₃ Pro.₃ Ser.₁ Tryp.₁ Tyr.₁ Val.₃. This would indicate a minimum molecular weight of about 3,300.

Landmann, Drake & White (91) have investigated the nature of the amino acid carrying the free α -amino group at the end of the peptide chain (NH₂- or N-terminus) of corticotropin A and substances derived from it by use of the dinitrofluorobenzene (DNFB) method of Sanger (93), together with a modification of the thiohydantoin method of Edman (94) whereby the hydantoin can be directly identified by paper chromatography (95). By this means it was found that corticotropin A yielded a single thiohydantoin corresponding to that of the amino acid serine (91). The stepwise degradation was continued by a second application of the Edman reaction, and again a single thiohydantoin was detected, this time that of tyrosine. Further application of the stepwise degradation technique gave equivocal results, but in confirmation of the deduction that the sequence at the N-terminus end of the peptide chain was Ser.Tyr., the dipeptide seryl-tyrosine was isolated from the products of the digestion of corticotropin A with chymotrypsin, its structure being assessed by a combination of enzyme hydrolysis, chromatography, and Sanger's DNFB method. As the authors point out (91) cleavage of the peptide chain of corticotropin A to form the depeptide Ser.Tyr. is consistent with the known specificity of chymotrypsin (96). Subsequently Hays & White (88) report the isolation, from prolonged peptic digestion of corticotropin A, of the tetrapeptide Ser.Tyr.Met.Glu., presumably an extension of the N-terminal sequence. Hays & White (88) find that the fission of a peptide linkage anywhere towards the N-terminus destroys biological activity; on the other hand fission towards the C terminus (free carboxyl group end of peptide chain) need not result in loss of activity. In particular White (92) has studied the nature of the amino acids released after various time intervals during the carboxypeptidase digestion of corticotropin A, the amino acids being identified chromatographically on paper with the system *S*-butyl alcohol/3 per cent ammonia. By photometric measurements of the ninhydrin (1,2,3-indantrione hydrate) colour in comparison with that from chromatograms with standard quantities of amino acids, quantitative values were obtained. By this means it was determined that the order of release was phenylalanine, glutamic acid, and leucine. Thus the C-

terminal amino acid is phenylalanine, and the sequence is Leu.Glu.Phe. Additional evidence for this sequence came from a study of the peptide fragments resulting from hydrolysis of corticotropin A with pepsin. These fragments were identified as Pro.Leu.Glu.Phe., Pro.Leu.Glu., and phenylalanine. From this it was deduced that corticotropin A has the C-terminal sequence, —Pro.Leu.Glu.Phe. and that on prolonged treatment carboxypeptidase splits off Leu., Glu., and Phe. quantitatively, stopping at proline, while pepsin splits off the entire tetrapeptide, and also apparently makes a secondary fission in the tetrapeptide, between its glutamic acid and phenylalanine.

Hays & White (88) suggest that the conversion of corticotropin A to corticotropin B entails splitting off the terminal acidic tetrapeptide, Pro.Leu.Glu.Phe., plus several other acidic fractions totalling 10 or 12 amino acid residues without loss of biological activity. There is possibly a rise of activity in the intravenous Sayers test from 100 to 125 I.U./mg. for corticotropin A to 150 I.U./mg. for corticotropin B, a rise which is consistent with the reduction in molecular weight. Utilizing the molar absorption coefficients in the ultraviolet, and assuming that no tyrosine or tryptophan is lost in the conversion of corticotropin A to corticotropin B, they find (88) that the relative absorption values indicate a reduction of approximately 25 per cent in molecular size. The isoelectric point of corticotropin A is about 7.5, while that of corticotropin B is approximately 10, and such a difference is in agreement with the belief that several acidic peptides are removed in the formation of corticotropin B from corticotropin A.

Hays & White (88) find that the biological activity of corticotropin A is destroyed by the action of tyrosinase, and that in general any process which leads to a diminution in the spectrophotometric absorption ascribed to tyrosine and tryptophan leads to a loss in biological activity.

At present the known properties of what are regarded by some as essentially pure corticotropins A and B do not agree sufficiently to make certain that a single corticotropin A has been obtained, giving rise to a single corticotropin B. For instance the figures for the amino acid content of corticotropin B (85) do not agree with those expected from the data available for corticotropin A (88, 91, 92). The differences in the biological activities claimed for preparations from different groups of workers are also alarmingly high, and outside of what might be reasonably expected from biological assays in which comparisons with an international standard preparation, or substandard, are made. Hays & White (88) state

A few words should be said regarding the wide differences in values for the physiological activities of these and other preparations referred to in the literature. In our laboratories, the highest values for corticotropin A have been 100–125 units/mg. and for corticotropin B 150 units/mg. while other groups report twice this value. Without pretending to know the reasons for these discrepancies, we would like to state that the same ratios seem to hold at low levels of purity on types of material which have been assayed by all groups. For instance, our values for commercial acid acetone powder are invariably in the range of 1.0 to 1.6 units/mg. while the Merck group

has reported a value of 3.5 units/mg. for such a fraction obtained from the Armour Laboratories. Again, our oxycellulose eluates are in the range of 25-40 units/mg. while Astwood reports a value of about 80 units/mg.

In general, it may be inferred that no final conclusions can yet be accepted about the properties of pure corticotropin A and corticotropin B, and the possibility must be entertained that the corticotropin A or the corticotropin B at present available is by no means a pure substance, or indeed that neither is pure.

A number of investigators have found various ACTH preparations to be unusually rich in zinc (97, 98, 99) and the observed high zinc content of whole pituitary glands has led to the suggestion that zinc may play a role in the release of the hormone from the gland of origin (97, 98). ACTH also binds zinc *in vitro*, and, for a preparation with a given history, the binding of zinc is found to be proportional to the ACTH activity (100); copper is bound similarly, and although manganese, cobalt and nickel are all bound by ACTH, no proportionality between binding capacity for these elements and biological activity was observed (100).

It would be expected that a peptide with the content of basic amino acids found in ACTH would bind zinc by association, and for the same reason the peptide might be expected to react with heparin. Considerations such as these are probably relevant to the observation that when heparin and ACTH are administered simultaneously to an animal the activity of the ACTH (101) and of heparin measured *in vitro* (102) is greatly depressed.

The stability of ACTH in vivo.—With the availability of purified preparations of corticotropin A for administration to human beings, a lack of correlation between activity in the Sayers test and in human beings became apparent for some though not all such preparations (23, 38, 39, 42, 88, 103, 104, 105). Astwood *et al.* (103) observed that when given intramuscularly to human beings 1 I.U. of purified ACTH (oxycellulose adsorbate from unhydrolyzed material, i.e., corticotropin A type) was as effective as 2 I.U. of crude ACTH (glacial acetic extract without oxycellulose adsorption). Evidence has now accumulated that crude preparations of ACTH, hydrolyzed or unhydrolyzed, and more purified preparations of the corticotropin B type, are particularly liable to inactivation at the site of injection when administered subcutaneously or intramuscularly (23, 42, 43, 88, 105 to 108). Forsham *et al.* (43, 105) emphasize that a preparation of corticotropin B, obtained by the partial hydrolysis of a preparation of corticotropin A, may be almost completely inactivated when administered intramuscularly, even though it retains full activity intravenously. "Thus, a small change in molecular configuration, consisting in decreasing the chain length of an ACTH polypeptide, appears to make the preparation susceptible to intramuscular inactivation in man" (105). On the other hand the greater susceptibility to intramuscular inactivation of crude unhydrolyzed preparations of ACTH as compared with preparations of ACTH purified by oxycellulose adsorption

and elution may result from the removal, during the oxycellulose step, of substances, present in the crude preparation, which aid destruction, possibly by tissue enzymes (108). The presence of systems in blood (109, 110) and in tissues (111, 112) which inactivate ACTH has been described, but why crude ACTH and corticotropin obtained by partial hydrolysis should be so much more susceptible than purified corticotropin A to extravascular inactivation is still far from clear. This problem has an important bearing on the standardization of ACTH since, if material is standardized by a Sayers test, which involves intravenous administration, and is then administered intramuscularly to humans, corticotropin of the A type may be perhaps two or three times as effective as that of the B type in the human subject (cf. 23, 88, 103, 108). It appears likely that in the future it may be deemed safer to standardize ACTH for clinical use by means of a test involving subcutaneous and not intravenous administration (23).

ACTH AND MELANOPHORE-EXPANDING ACTIVITY

The suggestion that ACTH is identical with the melanophore-expanding hormone ["intermedin," "blackening hormone" or "B" hormone of the *pars intermedia* (113)] was made by Johnsson & Hogberg (114) while Sulman, after first concluding that ACTH is intermedin (or a chromatophore hormone) (115, 116), later suggested that ACTH is a complex of three factors of which intermedin is but one (117 to 120). It is certainly true that the best preparations of ACTH so far obtained do possess substantial melanophore-expanding activity (85, 90, 121), but the treatment of an active preparation of ACTH by heating in strongly alkaline solution (0.1N NaOH for 5 to 10 min. at 100°C.) leads to a substantial loss of ACTH activity (Sayers test) with a maintenance or even a potentiation of melanophore-expanding action (122 to 126). However, as Waring & Ketterer (16) emphasize, it is unwise to assume that intermedin activity is stable to heating with alkali under all conditions. They point out (16) that material which has been prepared by adsorption on, and elution from oxycellulose, a process which would be expected to diminish the protein content of the extract, yields intermedin activity which is unstable to heating with alkali, and in which the loss of ACTH activity (Sayers test) and melanophore-expanding activity proceeds at the same rate under such destructive conditions. Although, in view of such evidence, an identity of the structures concerned with ACTH activity and intermedin activity is difficult to maintain, some relationship between them may nevertheless exist, particularly so if either hormone consists of more than one factor. Some evidence against a simple identity springs from the fact that melanophore-expanding activity greatly predominates over ACTH in extracts of the *pars intermedia* of the pituitary body, while ACTH is relatively higher in anterior lobe extracts (122, 123, 127).

Johnsson & Hogberg (128) observed that the ascorbic acid reducing activity of their ACTH is almost completely lost when it is heated at 100°C.

for 20 min. in 0.1N NaOH. They found that such treatment reduces melanophore-expanding activity by only 25 per cent while some eosinopenic action also remains in human subjects. The subjects treated with the alkali-heated material also showed an increased urinary excretion of potassium and of dehydroisoandrosterone. It is clear from their own observations, that Johnson & Hogberg (128) cannot maintain that a simple identity holds between ACTH (as measured in the Sayers test) and intermedin. Indeed, a conclusion from the observations in this publication (128) is that "an increased production of corticotrophin is accompanied by a corresponding rise in the melanophore hormone," the inference being that corticotropin and the melanophore hormone are not identical. The significance of the fall in eosinophil count which they observed to follow the administration of ACTH inactivated by heating in alkali is doubtful since the administration of many nonspecific proteins will induce eosinopenia (see 18, p. 28). The evidence which they adduce is not sufficient to warrant the assumption that ACTH retains physiologically significant activity after heating in alkali, except that of expanding melanophores.

The possibility cannot be excluded that more than one pituitary factor may be concerned with the control of chromatophore (including melanophore) expansion in different species, or even within the same species. Thus Astwood (129) found that while crude posterior pituitary extracts caused expansion of erythrophores, e.g., ventral reddening of *Phoxinus*, as well as melanophore expansion in frogs, treatment of the extract with alkali potentiated the melanophore-expanding activity of the extract while reducing its erythrophore-expanding activity to about one-tenth of its former value. Furthermore Lock (125) finds that when pig pituitary ACTH is heated for 10 min. at 100°C. in 0.1N NaOH the ACTH activity is almost completely destroyed, the chromatophore-expanding activity in *Hyla arborea* (the tree frog) is reduced about five-fold, while the melanophore-expanding activity in *Xenopus laevis* (African clawed toad which possesses only melanophores) is unchanged. Lock suggests that *Hyla* responds to both ACTH and intermedin, while *Xenopus* is affected by intermedin only.

Morris (124) claimed that intermedin can be obtained free from ACTH by the carbon adsorption method of Landgrebe, Reid & Waring (130) for the preparation of intermedin. Both Li and his colleagues (123) and Waring & Ketterer (16) disagree with this. Although some degree of separation of intermedin and ACTH activities can be effected (2, 131) complete separation has not been achieved, and the inference has to be considered that intermedin and ACTH (material active in the Sayers test) may be sufficiently chemically similar to overlap somewhat in biological activities. Some such degree of overlap has already been considered with respect to the chemically similar posterior pituitary factors, vasopressin and oxytocin (132). Such a similarity, both chemically and biologically might obtain with respect to intermedin and ACTH, but for such a speculative view there is at present no good evidence, although it would agree with the idea put forward by Sulman that in-

termedin is but one constituent of an ACTH complex (117 to 120). Clearly the observation that intermedin can influence carbohydrate metabolism by inhibiting the hyperglycaemia attributable to the administration of epinephrine or to stress (113, 133), could be interpreted as an ACTH-like action.

Evidence in support of a relationship between intermedin and ACTH has been based on the existence of excess of melanophore expanding material (117, 119, 128) in the blood of patients with Addison's disease, where high blood ACTH levels are to be expected because of a low blood adrenal corticosteroid content (134), and also in those with Cushing's syndrome, in pregnancy or exhibiting stress, where again a high blood content of ACTH might be expected (128, 135). Such evidence must necessarily be equivocal and may be contradictory in some instances (136), but it is clear that in pregnancy urine there is a melanophore-expanding substance with no obvious ACTH activity (137, 138). Indeed it has been claimed (139) that in human pregnancy urine and plasma there exists a substance which, in the frog *Rana esculenta*, expands the erythrophores of the hind limbs only, while ACTH affects the erythrophores over the whole body. Moreover, heating to 100°C. at pH 7 did not destroy the activity of substances like ACTH, which affected the whole body, but did inactivate those affecting the legs only (139, cf. also 140).

One must conclude that the specificity of melanophore- or erythrophore-expanding activity is not absolute and that the most likely explanation of the present confused picture is that ACTH itself does possess some melanophore-expanding activity. If this is so, then the clinical observations, including the fact that pigmentation similar to that observed in Addison's disease is sometimes found, following long treatment with ACTH in man (141), would find ready explanation.

THE PROPERTIES OF MATERIAL ACTIVE IN TESTS FOR ACTH OTHER THAN THE SAYERS TEST

Of the biological tests, other than the Sayers test, described above (page 406) the adrenal weight increasing or maintenance test is of particular interest, having been used for the first demonstration of ACTH activity in anterior pituitary extracts. Stack-Dunne & Young (78) and Dixon *et al.* (142) were the first to compare the activities of different pituitary preparations in the Sayers test and in the adrenal-repair test, and they inferred that at least two substances of anterior pituitary origin were concerned in the observed effects. In their view any substance originating in the anterior pituitary gland which influences the adrenal cortex, is eligible for the description "adrenocorticotrophic." In their experiments the adrenal-repair test was based on the gain in weight of the adrenal glands of a rat hypophysectomized one to three weeks previously and given three intraperitoneal injections of pituitary extract daily for three days, the animal being killed on the fourth day. Since an increase in adrenal weight does not measure only changes in the weight of active cortical tissue, such an effect is necessarily less precisely defined than an

ascorbic acid-reducing effect. In their investigations (78, 142) an Armour Laboratories preparation 85/85 U was taken as a standard for both adrenal weight increasing (A.W.²) and ascorbic acid reducing (A.A.²) activities, this preparation assaying about 2.5 I.U. per mg. in the Sayers test. In terms of this standard, with an A.W./A.A. ratio defined as unity, a crude alkaline extract of ox pituitary gland exhibited an A.W./A.A. ratio of approximately 2,000/1 to 5,000/1 while a preparation of ox pituitary growth hormone yielded a ratio of 800/1 (78). On the other hand an Armour laboratories preparation 84/85 H, obtained by a method involving partial hydrolysis with both pepsin and acid yielded a ratio of 1/8. Since the test employed for adrenal weight increasing activity involved the intraperitoneal administration of the material under test, and since preparation 85/85 H was partly of the corticotropin B type, some degree of extravascular inactivation may have occurred in this test and may have contributed to or accounted for the low A.W./A.A. ratio observed for this preparation. Indeed any argument which is based on a comparison of the activities of two different preparations in different tests, involving different routes of administration and perhaps yielding dose/response curves of different slopes, must be regarded with caution. Such caution must clearly be applied to any simple interpretation of the fact that on pepsin or acid hydrolysis ACTH preparations often lose a relatively greater degree of adrenal weight increasing or eosinophil reducing activity than of ascorbic acid reducing action (10, 11, 90, 143, 145). Such evidence alone would be dangerous on which to base an assumption that these different activities cannot be credited to a single ACTH. Hungerford, Reinhardt & Li (144) find that when ACTH is heated in 0.1 N NaOH for 10 min. at 100°C. adrenal-ascorbic acid reducing activity is drastically reduced, but the eosinophil reducing activity in hypophysectomized rats is unaltered. Again it must be emphasized that eosinopenia can be induced by nonspecific proteins (18, p. 28). As the result of finding a lack of correlation between the Sayers test and eosinopenic test for different preparations, Hungerford, Reinhardt & Li (144) tentatively conclude that there must be present "an eosinophilic component in certain ACTH preparations." The separate existence of eosinophil-reducing and ascorbic acid reducing components of ACTH at present appears to be unproven.

In the experiments of Dixon, Stack-Dunne & Young (78, 80, 142) the adrenal weight increasing activity of a crude alkaline extract of ox pituitary gland was substantial, though its activity in the Sayers test was very small indeed. The low activity of this extract in the Sayers test may have been caused by the presence of proteolytic enzymes in such a crude extract (112), which affected material active in the Sayers test much more than other factors. Winter, Brink & Folkers (121) investigated the activity of an alkaline extract of ox pituitary glands, having an ascorbic acid reducing activity of less than 1 I.U./mg., although no precise figures are given for the Sayers activity of this preparation. This extract possessed moderate adrenal-weight increasing activity, and the authors did not attempt to calculate A.W./A.A.

ratios for this or any other preparation investigated, because very different dose/response curves were obtained for different preparations. Nevertheless it was clear, from their experiments, that purified corticotropin B possessed a high degree of adrenal-weight increasing activity (121, see also 85). As the authors point out this does not prove that some substance other than corticotropin B might possess only adrenal-weight increasing activity, though it might be necessary to prove the absence of corticotropin B in other materials before a satisfactory conclusion could be drawn. As Stack-Dunne (80) has observed, an increase in adrenal-weight can be a result of hypertrophy of cells or engorgement of blood vessels in addition to hyperplasia, and it must therefore be expected that material active in the Sayers test would always show some activity in a test based on adrenal-weight. In such a case the histological picture might serve to demonstrate the difference between the two types of action. The administration of an A.W. preparation (growth hormone) of Dixon, Stack-Dunne & Young to hypophysectomized rats induced a histological state approaching normal, as judged by lipid distribution and also by mitotic activity (80, 142, 146). On the other hand any quantity of an A.A.² preparation (adrenal ascorbic acid reducing preparation) sufficient to induce an appreciable regeneration of cells also caused changes in the lipid distribution reminiscent of those observed in the normal animal under stress (80, 142, 146).

In an attempt to fractionate the A.W.² factor Dixon, Stack-Dunne, Young & Cater (142) employed the ion-exchange resin Amberlite IRC-50 and found that fractions active in the A.A. test also possessed A.W. activity but the fractions which ran faster on the column produced very large adrenal glands in the A.W. test although they showed only slight A.A. activity. In such instances it might be expected that A.A. active fractions would be more effective in an A.W. test when mixed with protein or other inert material such as would be present in the fractions running fast on the column, since the presence of such inert material might diminish the rate of absorption of the extract from the tissues in the A.W. test and thus enhance the biological effectiveness of the slowly absorbed material. It is known that the general effectiveness of ACTH preparations is enhanced by extravascular administration in a medium designed to delay absorption from the tissues (38, 147 to 152) almost certainly because of the short half life of ACTH in the circulation, about 1 min. for the rat (153). Stack-Dunne *et al.* (79, 80, 142) endeavoured to equalize the rates of absorption from the peritoneum in the adrenal-weight test by mixing the extracts just before injection with protein (serum albumin or casein) and then with tannic acid in sufficient amount to give maximum precipitation. The high and low A.W. activities were again observed with such treated extracts (80, 142); this was still true (80) when certain dry preparations were suspended in 5 per cent beeswax in arachis oil, according to the method of Bruce & Parkes (147), even though the activity of both highly active and slightly active A.W. fractions was then raised 20-fold. It seems most improbable that the effective-

ness of some preparations, as compared with others, in the A.W. test can be attributed mainly or entirely to an assumed slower rate of absorption of these preparations from the tissues of the test animal.

In the experiments of Stack-Dunne *et al.* (80, 142, 146) preparations of growth hormone were effective in the A.W. test, though no conclusion could be drawn as to whether or not growth hormone was itself the active agent. It should be recalled that Selye (154) has claimed that growth hormone possesses mineralo-corticotropic effects, but concludes that the presence of ACTH is necessary in order that growth hormone may bring about its mineralo-corticotropic effect and that growth hormone is unable to elicit its potential mineralo-corticotropic action after the adrenal cortex has become atrophic, either as a result of hypophysectomy or to excessive dosage with adrenal steroid (154). In contradiction of Selye's view, Stein *et al.* (155) have shown that the administration of growth hormone to adrenalectomized, as well as to normal, rats induces a retention of Na^+ , K^+ , and Cl^- . This was true for adrenalectomized rats whether or not they were treated with adrenal extract and the authors conclude that the effects of growth hormone on Na^+ , K^+ , and Cl^- are not mediated by the adrenal cortex. In the opinion of the reviewers there is no significant evidence that growth hormone is a mineralo-corticotropic substance.

Stack-Dunne (80) and Cater & Stack-Dunne (146) found that treatment of hypophysectomized rats with growth hormone led to a substantial stimulation of mitotic activity in the *zona glomerulosa* and the outer part of the *zona fasciculata*. This effect could not be duplicated with A.A. preparations. When A.A. preparations and growth hormone were given simultaneously there was some evidence of synergism in adrenal-weight increase, but none in the mitotic stimulating activity itself.

Bush (156) has reported that when A.A. and A.W. (growth hormone) preparations supplied by Stack-Dunne were injected intravenously into hypophysectomized rats and the adrenal venous blood assayed for corticosterone by paper chromatography, a great increase in the rate of secretion of corticosterone followed the injection of A.A. preparations, but not that of A.W. fractions. Pretreatment of the hypophysectomized rat by subcutaneous, intraperitoneal, or intravenous administration of the fractions all led to the same result. Since Winter *et al.* (48) have found that treatment with androgen plus cortisone largely repairs the atrophied adrenal glands of the hypophysectomized rat, it is possible that differences between the relative ascorbic-acid reducing and adrenal-weight increasing activities of different ACTH preparations might be ascribable to the relative proportions of C_{21} and C_{19} steroids secreted by the adrenal glands in response to ACTH stimulation under different conditions. Nevertheless, it would be difficult to accept such a possibility as an explanation of the fact that pituitary preparations with almost no A.A. activity are effective in A.W. tests (78, 142) in the absence of evidence that ACTH can induce the secretion of C_{21} steroids by the adrenal glands of the hypophysectomized rat, under the conditions of

the Sayers test, without causing a significant fall in adrenal ascorbic acid level.

Rinfret *et al.* (157) have prepared an extract of horse-pituitary gland which is poor in A.A. activity but which is active in maintaining the adrenal glands of hypophysectomized rats (A.W. preparation). Liddle and co-workers (158) have administered this and a similar extract of pig pituitary gland to human beings and find that the intravenous administration of such an extract, in contrast to that of conventional ACTH, led to almost no immediate increase in adrenal steroid secretion, as estimated by urinary 17-hydroxycorticoid and 17-ketosteroid excretion, or changes in circulating eosinophils. When a subject was treated with the adrenal-weight increasing fractions for five successive days the subsequent administration of a standard dose of conventional ACTH led to an increase in the urinary excretion of 17-hydroxycorticoids and 17-ketosteroids which was two or three times that consistently observed in the same subjects before treatment with the A.W. preparation. Purified conventional ACTH and growth hormone both failed to induce an effect similar to that obtained with the A.W. preparations used in these experiments (158).

Although lack of correlation has been observed between many of the activities ascribed to ACTH, for example those concerned with ascorbic-acid reduction, adrenal-weight increase or maintenance, eosinopenia and thymus atrophy, it is only with respect to the alleged differentiation between ascorbic-acid reducing and adrenal-weight increasing factors that there is any serious body of evidence. Here, however, it is clear that separable factors, each uncontaminated with the activity of the other, have not yet been obtained, and indeed may not be obtainable (cf. 121). Until further evidence is available the claim that an adrenal-weight increasing factor can be differentiated from ordinary ACTH, active in the Sayers test, must be regarded as unproven. The situation here may resemble that found with respect to oxytocin and vasopressin of the posterior pituitary where vasopressin appears to have intrinsic oxytocic activity as one of its biological activities (132) though oxytocin is not found to possess pressor activity. Such a possibility must be considered with respect to ACTH and intermedin (see above) and also with regard to adrenal weight increasing and ascorbic acid reducing factors; much further research is needed to establish such a possibility.

THE INFLUENCE OF ACTH ON THE ADRENAL GLAND

The nature of the steroids secreted by the adrenal glands.—Largely as a result of the development of elegant chromatographic methods, particularly by Bush (7, 159) and by Zaffaroni (160), the nature of the principal steroids secreted by the adrenal gland in many species has become clear. These are 17-hydroxycorticosterone and corticosterone (7, 159, 160, 161). The ratio of 17-hydroxycorticosterone to corticosterone varies with different species, the extreme values lying between $<20/1$ for the rhesus monkey and $<0.05/1$ for the rat (159). In any individual, changes in the total rate of secretion produce

no change in the relative amounts of the different components of the secretion according to Bush (159), though Bush & Sandberg (162) find that treatment of the human with ACTH tends to reduce the 17-hydroxycorticosterone/corticosterone ratio to about 4/1 if the ratio was previously above this.

The Porter-Silber reaction (163) has been widely used for the estimation of 17-hydroxycorticosteroids. Direct estimation by this means, on human adrenal vein blood obtained by catheterization, suggest that the human adrenal secretes 15 to 25 mg./day of 17-hydroxycorticosteroids (164). In peripheral human blood 3 to 25 $\mu\text{g.}/100$ ml. of 17-hydroxycorticosteroids are found (165, 166) while Morris & Williams (167) find for human plasma 6.5–10.5 $\mu\text{g.}/100$ ml. of 17-hydroxycorticosterone and 4 to 10.5 $\mu\text{g.}/100$ ml. of corticosterone. The values observed by Morris & Williams for corticosterone are substantially higher than those of Bush (159, 162) and of Nelson & Samuels (165) but agree with those of Sweat *et al.* (161).

In addition to 17-hydroxycorticosterone and corticosterone other adrenal steroids are found in blood in small amount. Bush (159) found evidence for the secretion of 11-hydroxy- Δ^4 -androstene-3,17-dione in many species, while Zaffaroni & Burton (160) observed small quantities of seven unidentified substances giving the reactions of an α -ketol in the adrenal vein blood of the dog. Morris & Williams (167) observed reactions for significant amounts of 11-dehydrocorticosterone and of cortisone in human plasma, and suggest that these 11 keto substances may result from the oxidation of 11-hydroxycorticosteroids in the tissues.

Balfour (168) found that in the calf less than two days old, the major steroid components of adrenal-vein blood were two members of the "amorphous fraction" together with 17-hydroxycorticosterone. In foetal lambs the major component was also a member of the "amorphous fraction." After two days of age the "amorphous fraction" substances were no longer major constituents while 17-hydroxycorticosterone continued to be present. Corticosterone first appeared in adrenal vein blood at about 10 days after birth. Balfour (168) also observed that when ACTH was given to calves less than 8 days old there was no increase in steroid output. The effect of ACTH in calves from 8 to 40 days of age was to increase the total output of steroid not by increasing the concentration of hormones in the blood, but by increasing only the rate of circulation of blood through the gland. An increase in adrenal blood flow induced by ACTH has also been observed by de Guirpide (169) in the dog.

There is a growing body of evidence that the isolated and characterized adrenal steroids do not account for all the actions of crude adrenal cortex extract (e.g., 170). Tait, Simpson & Grundy (171, 173), developed a method for assay of adrenal mineralo-corticoids based on the lowering of urinary $\text{Na}^{24}/\text{K}^{42}$ ratio in adrenalectomized rats in a 2 hr. interval after the injection of the radioactive isotopes. They found that a substance behaving chromatographically like cortisone but with very high mineralo-corticoid action was present in crude adrenal extract. A substance having similar properties was

also present in the adrenal venous blood of the monkey and the dog (172). Subsequent studies (174, 176) made clear that a new steroid was responsible for these effects, and Simpson *et al.* (175) have recently announced its isolation, though the details of its structure are as yet unpublished. It appears to be a C_{21} steroid and a Δ^4 α - β unsaturated ketone, with α -ketol side chain at C_{17} , and an additional hydroxyl group apparently not 17α , 16α , or β , nor 12β (175, 176). It is much more active than 11-deoxycorticosterone in mineralocorticoid properties, the ratio of activities between the new steroid and deoxycorticosterone varying, according to the method used for comparison, between 30/1 and 100/1 (175). The name "electrocortin" has been suggested for the new steroid because of its high activity on electrolyte balance (176). Mattox *et al.* (177) have recently isolated a similar substance from ox adrenal extract and provide evidence that the unlocated hydroxyl group is probably not at position 6. Knauff, Nielson & Haines (178) have also obtained a similar substance from pig adrenal extract and find that it has little or no activity in the glycogen-deposition or eosinophil-depletion tests for adrenal steroids. A lack of effect on glycogen deposition would agree with the absence of a hydroxyl group from position 11. Farrell & Richards (179) have confirmed the findings of Simpson, Tait & Bush (172) with respect to the presence of electrocortin in the adrenal vein blood of the dog. Since all concerned are agreed that the new steroid does not give a Porter-Silber test (176 to 179) the presence of a 17α hydroxyl group appears to be excluded.

The isolation of electrocortin appears to have opened up a new and important approach to the biochemistry of adrenal steroids and may require the revision of many of the presently accepted views.

ACTH and urinary steroids.—Although the administration of ACTH is followed by a rise in the urinary excretion of 17-hydroxycorticosteroids (26, 27, 28) it is clear that most of the steroids secreted by the adrenal glands undergo degradation in the tissues and are excreted in the urine in many different forms (180, 181). There is no doubt that the C_{17} α ketol side chain of adrenal steroids can be rapidly degraded in tissues (182). Reduction of the conjugated unsaturated system can also occur without degradation of the side chain, leading to various hydroxy and keto allopregnan derivatives (183). The catalogue of urinary metabolites of adrenal steroids is by no means exhausted yet and will provide problems for teams of research workers for a few years more.

The observations of Sprechler & Vesterdal (184), that ACTH induces a much smaller increase in 17-ketosteroids than in corticosteroids in children, in comparison with the effect of ACTH in adults, may be considered in conjunction with those of Balfour (168) on the young calf, to indicate that age may play a bigger part in determining the type of adrenal steroids secreted, and of the corticosteroid metabolites excreted, than has hitherto been considered.

Salamon & Dobriner (185) have isolated a steroid of adrenal origin, 11 β -hydroxy- Δ^4 -androstene-3,17-dione (cf. also 159) from the urine of patients

receiving therapeutic amounts of ACTH. This substance was not found in the urine of normal subjects, nor of those treated with large amounts of cortisone. The authors believe that the α - β unsaturated ketone structure in ring A indicates that this substance is secreted as a primary hormone by the adrenal gland and in part is excreted without transformation to other metabolites. They believe that it may be an important constituent of the "adrenal androgens" and, as such, may play a significant role in the metabolic response to adrenal secretion. The claimed increased urinary excretion of dehydroepiandrosterone after ACTH treatment in the human (186) is of particular interest since this substance is excreted in large amounts by patients with adrenal tumours, but not by those with adrenal hyperplasia (187, 188, 189).

Metabolism of steroids in adrenal tissue.—Pincus, Hechter, and their colleagues have summarized their experiments demonstrating that the isolated perfused ox adrenal gland possesses a system which introduces an oxygen atom at position 11 β in the steroid nucleus (190). Their paper illustrates the production of corticosterone from deoxycorticosterone on a laboratory scale by this technique. Similarly 17-hydroxy-11-deoxycorticosterone and its 21-acetate have been oxidized to 17-hydroxycorticosterone (191), while Δ^4 -androstene-3,17-dione was analogously converted to 11 β -hydroxy- Δ^4 -androstene-3,17-dione (192). In the latter case some 11 β hydroxyandrostane 3,17-dione was also formed, which indicates that reduction of the double bond at 4:5 could also occur. Multicycle perfusion of Δ^4 -androstene-3 β -ol-17-one (dehydroepiandrosterone) led to two transformation products, only one of which could be characterized, this being Δ^4 -androstene-11 β -ol-3,17-dione (193). Perfusion with cortisone led to the isolation of allopregnan-3 β ,17 α ,21-triol-11,20-dione [tetrahydro-cortisone (3 β ,allo)] and allopregnan-17 α ,21-diol-3,11,20-trione (allodihydrocortisone) (194). The reduction of the α - β unsaturated ketone grouping which occurred in these experiments also took place when cortisone (or adrenosterone) was incubated with ox blood (194). After a multicycle perfusion of androstan-3 β -ol-17-one (epiandrosterone) three transformation products were identified (195). In order of diminishing yields these were androstan-3,17-dione, androstan-11 β -ol-3,17-dione, and androstan-3 β ,11 β -diol-17-one. The oxidation of the 3 β hydroxyl group to 3-keto, which had occurred in addition to the introduction of a hydroxyl group at 11, was found in reverse when allopregnan-21-ol-3,20-dione (a rat liver metabolite of deoxycorticosterone) was perfused, since the major product was allopregnan-3 α ,21-diol-20-one, with smaller amounts of allopregnan 11 β ,21 diol-3,20-dione (196). In general this interesting series of papers has demonstrated the outstanding ability of the perfused adrenal gland to introduce an 11 β -hydroxyl group into a variety of steroids, while oxidation of a 3 β -hydroxyl group to 3-keto also can occur, both of these reactions being a necessary step in the conversion of cholesterol to corticosterone or 17-hydroxycorticosterone.

Haines (197) has reviewed the biosynthesis of adrenal cortex hormones and has shown that when a pig adrenal mince preparation is incubated with deoxycorticosterone, there can be isolated corticosterone, 11-dehydrocorticosterone, and 6 β -hydroxy-11-deoxycorticosterone; incubation of 17-hydroxy-11-deoxycorticosterone led to 17-hydroxycorticosterone and to cortisone. Again the introduction of an 11 β -hydroxyl group may have preceded an oxidation of the hydroxyl group to ketone.

In a most interesting series of papers Peterson, Murray, and their colleagues have examined (198 to 204) the microbiological transformations of steroids, particularly the introduction of oxygen at C₁₁. Here, interestingly enough an 11 α -hydroxyl group is the rule, in contrast to the 11 β -hydroxyl introduced by adrenal enzymes.

Hayano & Dorfman (205) have described the preparation of a washed adrenal homogenate which, when supplemented with fumarate and also if necessary with triphosphopyridine nucleotide (206), possesses a powerful 11 β -hydroxylating system. The isolation and identification of corticosterone, 17-hydroxycorticosterone, allopregnan-11 β ,21-diol-3,20-dione, and Δ^4 -androstene-11 β -ol-3,17-dione after incubation with their respective 11-deoxy derivatives, was effected. The authors propose the name 11 β -hydroxylase for the enzyme system catalyzing the C-11- β hydroxylation of certain C₂₁ and C₁₉ steroids. It is interesting that the 11-hydroxylase activity of the adrenal medulla is almost as high as that of the cortex (205). With adrenal slices (207) adrenal steroids are converted to more polar substances, probably by nuclear hydroxylation. Such substances, which possess a Δ^4 -3 keto-17 α ketol structure, may possibly be of the type occurring in the amorphous fraction of adrenal extracts (207). Hayano & Dorfman have not further defined the conditions for activity of the enzyme system in adrenal homogenates which they observed (208) to be capable of effecting oxidation of a C₂₁ methyl group to a primary hydroxyl group. In their earlier experiments simultaneous hydroxylation at C₁₁ and C₂₁ occurred with, for example, the conversion of progesterone to corticosterone (208). More recently (205) they have isolated radioactive corticosterone after incubation of their homogenate system with radioactive progesterone.

Plager & Samuels (209) have described the presence of enzyme systems, in the supernatant fraction of an ox adrenal homogenate, which will oxidize the C₂₁ methyl group of progesterone to a primary alcoholic group and introduce a 17-hydroxyl group into the progesterone molecule. The conversion of progesterone to 17-hydroxy-11-deoxycorticosterone was demonstrated with C¹⁴ labeled progesterone. The conversion is activated by adenosine triphosphate, by diphosphopyridine nucleotide or by triphosphopyridine nucleotide. The further conversion of the 17-hydroxy-11-deoxycorticosterone to 17-hydroxycorticosterone by incubation with ox adrenal mitochondrial preparations is also described (208). The pattern of enzyme systems thus far revealed demonstrates the later stages in the conversion of precursors such as

cholesterol to adrenal steroids but as yet throw no light on the earlier steps in this transformation.

The influence of ACTH on adrenal metabolism.—Safran, Grad & Bayliss (35, 36) have demonstrated that the production of corticosteroid *in vitro* by adrenal slices, or of whole rat adrenals, can be stimulated by the addition of ACTH *in vitro*. Haynes, Savard & Dorfman (33, 210) have shown that adrenal slices from pig or ox glands, incubated *in vitro* with acetate labeled with C^{14} in the carboxyl group, synthesize radioactive adrenosterone (210) and 17-hydroxycorticosterone (33) and that this process is stimulated by the addition of ACTH. More recently they have shown (34) that adrenal slices synthesize formaldehydogenic steroids *in vitro*, including corticosterone, 17-hydroxycorticosterone, and cortisone. This production is not enhanced by the addition of glucose, acetate, or fumarate, but the addition of ACTH causes a 2 to 6 fold increase over the original. Hofmann & Davison (211) find that with rat adrenal glands *in vitro* steroid production is increased by the addition of acetate, acetoacetate, or glycerol. Addition of ACTH also induced an increase in the absence of such substrates, but in their presence ACTH caused no further rise.

These *in vitro* observations as yet yield no conclusive evidence as to the step (or steps) in the process of corticosteroidgenesis at which ACTH can act to enhance steroid production. Hechter and his colleagues (212) have shown that ACTH does not stimulate the rate of incorporation of C^{14} from C^{14} -labeled progesterone into 17-hydroxycorticosterone or cortisone in a perfused ox adrenal. It may be that exogenous progesterone does not completely mix with the hypothetical intracellular intermediary progesterone, but it is more likely that ACTH acts at a point preceding the formation of the intermediary progesterone (212).

It is known that patients with congenital adrenalcortical hyperplasia often exhibit pseudohermaphroditism or macrogenitosomia praecox, with signs of adrenal insufficiency. Treatment with cortisone is usually beneficial, relieving the adrenal insufficiency and diminishing the signs of sex hormone activity and the excretion of 17-ketosteroids (213, 214). In such patients administration of ACTH did not induce eosinopenia, retention of salt, or loss of potassium (213, 214) but did, nevertheless, produce an increase in the excretion of 17-ketosteroids (214). An abnormally high blood ACTH (213) and the presence in the urine of a substance which is active in the Sayers test and which is not found in the urine of normal adults (214) has been reported in such patients. It seems likely that in these people there is a deficiency of production of those C_{21} adrenal steroids which might be expected both to maintain a normal electrolyte balance and to depress the secretion of ACTH by the pituitary gland. It is unfortunate that much of our present knowledge concerning the depression of ACTH secretion by the anterior pituitary gland is derived from experiments with 11-deoxycorticosterone and with cortisone, neither of which is a major secretory product of the adrenal gland (159, 160).

It may be that electrocortin, or an unidentified substance in the amorphous fraction, has a much greater effect in depressing ACTH secretion than known steroids, and that the deficiency of such an active steroid may be of particular significance in congenital adrenal hyperplasia. It appears that there is a deficiency, probably congenital, in the mechanism for the formation of C_{21} steroids, while the production of adrenogenic substances, presumably C_{19} steroids, can proceed at the high rate consonant with the high undamped blood ACTH levels. From such considerations it again appears that the action of ACTH must be related to an early point in the production of steroids, certainly a point which the production of C_{21} and C_{19} steroids share in common. Talbot *et al.* (215) have suggested that the human pituitary gland secretes two ACTHs, one concerned with 11,17-oxycorticosteroid production, and the other with 17-ketosteroid formation. An over-production of the latter factor, if it exists, might explain many of the findings in patients with congenital adrenalcortical hyperplasia, but at present there is little concrete evidence for the existence of such a separate pituitary secretion influencing primarily 17-ketosteroid production.

It is now well established that under suitable conditions the addition of ACTH to adrenal homogenates or to slices *in vitro* can induce an increase in oxygen consumption (29 to 32) and this phenomenon has been utilized for the development of an *in vitro* ACTH assay. Since ACTH rendered almost inactive in the Sayers test may be almost fully active in an *in vitro* system (29) caution is needed in the utilization of such novel assay methods. Knowledge of the enzyme systems involved is not yet at a stage where anything definite can be affirmed about the point at which ACTH acts in these systems.

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CLINICAL APPLICATIONS OF BIOCHEMISTRY^{1,2}

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In previous reviews the subjects of calcium-phosphorus metabolism, salt and water balance in relation to glandular function and disease, and some aspects of carbohydrate metabolism were considered in 1936; haemoglobin, proteins, nonprotein nitrogen constituents of blood, lipids, bilirubin, iodine, blood volume, jaundice, and digestion in 1940; and potassium, the phosphatases, blood iodine, and liver function in 1948. In the present review we shall deal with recent work on the precision of analytical procedures in the clinical laboratory, sodium and potassium, isotopic iodine in the investigation of the thyroid, and amino acids in the urine.

PRECISION IN THE CLINICAL LABORATORY

There is no doubt that the load on the clinical chemistry laboratory is increasing as more and more specimens are sent for analysis. Not only is there a greater burden in the quantitative sense, but there is also a continual pressure to extend the range of analyses performed. Clinical chemistry has always drawn its methods from other branches of analytical science, and pathologists have not been slow to apply spectrophotometry and electrophoresis to their own fields—to instance only two of the recent physical methods of analysis. It follows that the clinical chemist and his assistants must be much more skilled in techniques than they once were; in short, they must be better analytical chemists.

Inevitably this has altered the pattern of responsibility for the care of the patient. Clinical chemistry used to be done by persons (frequently medically-qualified) who knew the patient and his clinical picture. They were able to detect inconsistencies in their results and to check them. Nowadays, a large hospital laboratory analyses specimens in batches; the work is often supervised by a nonmedical chemist, and there may be little or no contact between patient and analyst.

With such mass-produced analyses, the possibilities of unsuspected error are great. Several surveys of the situation have been published (1 to 4), and we know of a number of other investigations which have not been reported. In most cases, samples of blood, plasma, serum, or prepared solution were sent to a number of different laboratories for analysis. Comparison of the results is usually a disquieting experience because for most constituents the agreement between laboratories is poor; for a few it is almost too bad to be

¹ The survey of the literature pertaining to this review was completed in September, 1953.

² The following abbreviations are used: ACTH for adrenocorticotropin; DCA for desoxycorticosterone acetate.

believed. Of course, it must be admitted that comparisons of this sort paint the worst possible picture. It is well known that agreement within one laboratory is comparatively easy to attain, while agreement between different laboratories is relatively much more difficult. The reasons for this are not well known, but it may be relevant to mention that laboratories both in Europe and America have failed to agree even in a simple measurement of optical density by spectrophotometry (5, 6).

It may be, then, that an individual laboratory produces results which are consistent and therefore clinically useful, although it disagrees with the results of other laboratories. This situation is not, of course, satisfactory, if only because it makes impossible a comparison of figures from different hospitals, a need which is becoming greater with increase in nutritional surveys and the laborious collection of data on normals (7, 8).

There are two measures which will improve the uniformity of results. In the first place, most clinical methods are not specific so that altering the method will alter, to some extent, the values obtained. This source of difference could be eliminated if laboratories adopted standard methods, such as those which have recently been recommended for most of the known constituents (9, 10) and, particularly, for 17-ketosteroids (11) and haemoglobin (12). This policy brings its own difficulties, since it is obviously undesirable to stifle initiative in modifying old methods and trying out new ones, and there is no doubt that different circumstances demand different methods.

The other useful measure is to treat the laboratory as if it were a factory producing components to a specified tolerance. At definite intervals, a solution, of composition unknown to the technician concerned, is analysed in a routine fashion, and the result obtained is compared with the correct value. It is appropriate to plot the results on a control chart as suggested by Wernimont (13), and this application has been described from several laboratories (8, 14, 15). It need not involve a great deal of extra work.

Although help in maintaining desirable standards of accuracy is available in the ways indicated, it cannot be denied that, fundamentally, the important things are the training and conscientiousness of the analyst himself. Much benefit will also be derived from friendly co-operation between laboratories.

PLASMA SODIUM AND POTASSIUM

Determination of the concentration of serum sodium and potassium by means of the flame photometer is now almost a universal procedure in clinical biochemistry. Modern views of the significance of abnormal concentrations of these cations in serum are briefly reviewed.

Sodium.—The normal concentration of serum sodium varies from 133 to 152 m. eq./l., 98 per cent of observations falling within this range (8). This value is maintained by the neurohypophyseal and renal homeostatic mechanism (16) and by the instinct of thirst (17). Any increase of body-fluid tonicity stimulates both thirst and the secretion of the posterior pituitary anti-

diuretic hormone; and, vice versa, a decrease of tonicity is inhibitory to both mechanisms. Sodium forms about 91 per cent of the total base of serum and extracellular fluid, and is therefore the main determinant of its tonicity. In diabetic ketosis, however, the increased blood sugar may contribute a significant fraction of the osmolarity of extracellular fluid, and serum sodium is consequently reduced (18).

Studies of experimental salt-depletion (19) have shown that slight or moderate depletion results in a diminution of extracellular-fluid volume, with sodium concentration maintained at normal levels. It is only in fairly severe depletion that reduced levels of serum sodium are found. Therapeutic restriction of dietary sodium is now commonly reinforced by the use of cation-exchange resins. These are chiefly effective in absorbing ingested sodium, the amount exchanged falling with low-sodium diets (20). Faecal sodium loss may, however, exceed that in the diet by as much as 50 m. eq./day, showing that some sodium may be taken up by the resin from gastrointestinal secretions (21). Significant hyponatraemia, after seven weeks of resin therapy, has been described (22).

Clinical salt depletion is more commonly attributable to abnormal losses from the body than to dietary depletion. Loss from the gastrointestinal tract by vomiting, diarrhoea, or discharge from fistulae has been well recognized for many years. Depletion attributable to excess sweating in tropical conditions became familiar during the second World War, and was often severe enough to produce definite hyponatraemia (23). Recently, more attention has been paid to excess sodium loss in the urine. This may occur in renal failure, in adrenal insufficiency, and following the administration of various diuretics, especially mercurials. In chronic uraemia, reabsorption of sodium by the renal tubules is reduced in amount with consequent excess urinary loss (24). Nickel *et al.* (25) have shown that this may result in a vicious circle, in that the sodium depletion may further reduce the glomerular filtration rate and cause increased urea retention. In certain rare forms of nephritis, the so-called salt-losing type described by Thorn *et al.* (26), the tendency to sodium loss is more extreme, and a syndrome very similar to Addison's disease is produced. There is a greatly reduced serum sodium and circulatory collapse. This is unaffected by DCA² therapy, but is improved by a high-sodium diet. Several recent clinical studies of this condition have been made (27, 28, 29). Enticknap (30), in a study of the pathology of the condition, considered that the most important aetiology was chronic pyelonephritis, but it is probable that other varieties of renal disease may cause this type of functional abnormality provided that there is mainly tubular damage.

The effect of adrenal hormones on electrolyte excretion has been further studied by Fourman *et al.* (31, 32). Whilst DCA mainly resulted in sodium retention, ACTH² and compound F (hydrocortisone) caused a loss of potassium which was at least as important as sodium retention. Cortisone, although correcting many metabolic defects in Addison's disease, e.g., reduced gluconeogenesis and abnormal water metabolism, is not sufficient alone to

correct the abnormal urinary sodium loss (33) and needs the addition of DCA². Electrolyte abnormalities similar to those of Addison's disease have been described in some cases of the adrenogenital syndrome (34), and again both DCA and cortisone were necessary in treatment. The specific salt-retaining steroid actually produced by the adrenal cortex has been separated from cortical extracts and adrenal venous blood by chromatographic methods (35, 36), and has now been isolated in crystalline form by (37). It is claimed to be 16-hydroxy-deoxycorticosterone and to be 30 to 100 times as active as DCA with respect to sodium retention.

Osmotic diuresis causes enhanced loss of urinary sodium, whatever the loading solute. Glucose, as an osmotic diuretic, has been studied in detail by Brodsky *et al.* (38). Sodium depletion, resulting from glycosuria in diabetic coma, averages 500 m. eq. (39). Platt (40) has given evidence that sodium loss in renal failure may in fact be attributable to osmotic diuresis with urea as the loading solute. The diuresis produced by mercurial injections has been shown to be entirely a result of the osmotic effects of increased sodium chloride excretion (41).

Schroeder (42) first drew attention to the low-sodium state induced by too vigorous sodium depletion in oedematous conditions, especially in congestive heart failure. More than one method of depletion is usually necessary to cause significant hyponatraemia. Thus, in congestive heart failure, a combination of the excessive use of mercurial diuretics and dietary sodium restriction, with or without the addition of cation-exchange resins, is the usual cause. Serum sodium tends to be definitely lower in cases of heart failure that have been treated with mercurial diuretics than in untreated cases (43). Similar low-sodium states may occur in hepatic cirrhosis, especially after abdominal paracentesis (44). Rather paradoxically oedema persists despite gross sodium loss and may only be relieved after infusion of hypertonic saline (45). Clinical features associated with the "low-salt syndrome" are a state of resistance to the diuretic action of mercurial injections and a reduced glomerular filtration rate with urea retention. These are usually restored to normal following saline infusion.

This "low-salt syndrome," attributable to excess sodium loss, must be carefully distinguished from "dilution hyponatraemia" (46), in which the primary abnormality is excess water retention. In this condition the osmoreceptors appear to be "set" at a lower level of tonicity, so that continued secretion of antidiuretic hormone occurs despite a low serum osmotic pressure. There are no dramatic associated clinical symptoms and signs, and, in particular, no evidence of circulatory collapse or urea retention. Infusion of hypertonic saline is of no benefit and merely increases oedema. Thirst occurs at serum levels of sodium which are still in the hyponatraemic range, and the equilibrium level of serum sodium returns to precisely the same as that before infusion. This condition may occur in late stages of any chronic debilitating disease associated with wasting of body tissue. It has been described in congestive heart failure (45), cirrhosis of the liver, pulmonary tuberculosis

(47), and tuberculous meningitis (48, 49). If the underlying condition can be brought under control, e.g., by effective streptomycin therapy in tuberculous meningitis, the serum sodium reverts to normal levels. If, however, no effective therapy is possible, dilution hyponatraemia, despite its lack of clinical effect, is of grave prognostic import. The hypotonicity of extracellular fluid is presumably secondary to similar changes within the body cells, and indicates profound disturbance of metabolic processes. There is usually no difficulty in distinguishing these two types of hyponatraemia, but confusion may arise where there is associated primary renal disease resulting in urea retention. Thus there is almost invariably some hyponatraemia in the oliguric phase of acute renal tubular necrosis (50). The general consensus of opinion is that this is a dilution hyponatraemia, and, therefore, there is no indication for hypertonic sodium infusions.

The opposite situation of hypernatraemia is much less common, since this abnormality tends to be rapidly corrected by stimulation of the osmo-receptors and by intense thirst. In states involving impairment of consciousness, however, the thirst mechanism may be in abeyance, and the neurohypophyseal homeostatic mechanism may be impaired. States of high serum sodium with low urinary sodium have been described after head injury (51), intracerebral neoplasms (52), and other conditions involving depression of consciousness (53).

Potassium.—The normal concentration of serum potassium varies from 3.5 to 5.6 m. eq./l., 98 per cent of observations falling within this range (8). The coefficient of variation is clearly much larger than that of sodium, the homeostatic mechanism being less delicate. Recent studies of experimental potassium depletion have helped in the interpretation of the causes of variation of serum potassium in disease. Black & Milne (54) have reported on uncomplicated dietary depletion over a short period, whilst Bland & Bassett (55) have extended these observations to a much more prolonged period of depletion. The experiments of Fourman & Ainley-Walker (56) were complicated by the fact that a coincident acidosis was produced by ingestion of cation-exchange resin in the ammonium cycle. Serum potassium rapidly falls to low normal levels, but values below 3.0 m. eq./l. are not easily obtained. It has been shown (57, 58) that, during removal of extracellular potassium by dialysis with the artificial kidney, potassium continually passes from the intracellular to the extracellular compartment. Similarly, during dietary depletion, extracellular deficits are continually being replenished from cellular reserves. Uncomplicated potassium depletion is associated with retention of sodium and consequent increase of the extracellular compartment, and also with the development of extracellular alkalosis. The latter leads to the familiar clinical state of hypokalaemic alkalosis. It can be safely stated that if potassium depletion is associated with an extracellular acidosis, e.g., in diabetic ketosis, in renal failure, or as a result of the effect of exchange resins, a complicating acidogenic factor is present. Balance data (54, 59) suggest that the development of the alkalosis is independent of any renal influence and is

secondary to passage of sodium and hydrogen ion from the extracellular to the intracellular compartment in exchange for potassium. This has been elegantly proven by Orloff *et al.* (60), who showed that the alkalosis of potassium deficiency could be restored to normal by potassium administration in the nephrectomized rat. It appears, therefore, that the increase of serum bicarbonate and serum pH in potassium deficiency is not indicative of any gain of alkali or loss of acid from the body. The extracellular alkalosis is associated with an intracellular acidosis attributable to an increased concentration gradient of hydrogen ion across the cell membrane.

In general a low level of serum potassium indicates a state of potassium depletion, but the correlation is by no means strict, and there may be considerable depletion though the serum potassium remains normal or high. This is particularly well seen in diabetic ketosis (61) and in abnormal loss from the gastrointestinal tract (62). The explanation is that in these clinical states there is a complicating sodium depletion with contraction of the extracellular compartment. It is only when this has been restored to normal by saline infusion that a low serum potassium is found. Whilst a reduction of serum potassium indicates in general that a state of potassium depletion is present it can give no indication of its magnitude. Progressive potassium loss may be occurring whilst serum levels are being maintained at low normal values from intracellular reserves. The actual degree of depletion can only be assessed by the isotope-dilution method (63) or by balance data during the restoration of the deficit.

Clinical depletion of potassium from reduced intake is seen as a complication of the treatment of oedematous states by cation-exchange resins. Potassium is taken up by resins preferentially to sodium and depletion is partly avoided by giving a portion of the resin in the potassium cycle. If this proportion is too large the efficacy of the treatment is reduced, since much less sodium is taken up by resin combined with potassium than if combined with ammonium or hydrogen ion (64). Significant hypokalaemia has been reported after 18 weeks of resin therapy (21).

Hypokalaemia is, however, much more commonly a result of abnormal loss of potassium in gastrointestinal secretions or in the urine. The importance of potassium depletion after abnormal fluid loss from the gastrointestinal tract in surgical patients has been reviewed by Lans *et al.* (65). Vomiting appears to be particularly liable to cause depletion (66), since gastric juice has the highest potassium content of all gastrointestinal secretions (67). Considerable loss may, however, occur in any state of severe or prolonged diarrhoea, and has been described in cholera (68), steatorrhoea (69), ulcerative colitis (70, 71), and overdose of laxatives (72). Studies of potassium loss in the postoperative state (73) suggest that there may be a tendency to excess urinary loss, secondary to a "stress" state with increased secretion of adrenocortical hormones, in addition to losses from the gastrointestinal tract. A preliminary report (74) of postoperative patients on a low-potassium diet indicates that excessive loss of potassium through the kidney is not a con-

stant feature, since renal retention of potassium was as efficient as in the normal subject.

There has recently been considerable interest in abnormal losses of potassium in the urine. The normal kidney does not appear to conserve potassium as efficiently as sodium. Thus, in a recent study of dietary depletion (55) on 14 m. eq. potassium per day, the urinary loss, after two months, was still greater than the intake. A patient, after a similar period on the Kempner rice-fruit diet (containing about 7 m. eq. sodium per day), will excrete less than 0.5 m. eq. sodium per day in the urine (75). In renal disease, powers of potassium conservation are further reduced, and there is a tendency to potassium deficit if the urinary volume is well maintained. It has been shown that, even in terminal uraemia, the kidney is capable of adequate excretion of potassium by a process of ionic exchange for sodium (24, 25, 76), the clearance of potassium being considerably higher than that of inulin. Although potassium depletion, sufficient to give rise to paralytic symptoms, may occur in ordinary renal failure (77), where there is comparable reduction of both glomerular and tubular function, the depletion is more likely to be found in the more uncommon varieties of failure with a predominant loss of tubular function. Thus, hypokalaemia with paralysis is not unusual in cases of renal tubular acidosis (78, 79) and has been described in the proximal tubular failure of the de Toni-Fanconi syndrome, both in the infantile (80) and the adult (79) forms of the disease. Severe hypokalaemia also occurs in the early diuretic phase of acute renal tubular necrosis, where improvement of glomerular function tends to precede that of the tubules (81, 82).

In diabetic ketosis prior to treatment, the serum potassium is usually normal or high (61) despite considerable potassium depletion secondary to the osmotic diuretic effect of glycosuria. Serum potassium becomes abnormally low during the first 18 hr. of treatment, as a result of the entry of potassium into cells from glycogen synthesis, further loss of potassium in the urine from osmotic diuresis, and expansion of the extracellular space from saline infusions. A detailed study of electrolyte balance during the treatment of diabetic ketosis has been reported by Nabarro *et al.* (39), who found an average potassium deficit of 350 m. eq. Low serum potassium has also been shown to occur in the postgastrectomy "dumping" syndrome from rapid absorption of carbohydrate (83).

A high serum potassium is less common owing to the efficiency of the kidney in the excretion of this cation. Both the normal and the diseased kidney are capable of excreting potassium by a process of ion exchange for sodium in the distal tubular lumen (84). A potassium clearance considerably higher than that of inulin has been described in the normal kidney after potassium infusions (85), in uraemia (24, 25, 76), in the early diuretic phase of acute renal tubular necrosis (86), and in alkalosis (87). This efficient homeostatic mechanism fails, however, if there is severe oliguria. The administration of potassium salts by mouth is safe, unless there is oliguria with or without surgical shock, or adrenal failure. Potassium infusions may cause

death from cardiac arrest if the rate of infusion is too high; a concentration of potassium of up to 40 m. eq./l. given not more rapidly than one litre in 3 hr., has been stated to be safe (88). The concentration of potassium in the right auricle and ventricle is the limiting factor of safety, since death has been recorded from local hyperkalaemia in the right heart, whilst general venous blood still had a low potassium level (89). High concentrations of potassium may be found in plasma of stored blood as a result of transfer of potassium from erythrocytes, and this may be a source of danger in rapid and massive transfusions (90, 91).

Spontaneous hyperkalaemia may occur in renal failure with severe oliguria, as in acute renal tubular necrosis and renal cortical necrosis. Potassium transfer from the cells to the extracellular space takes place as cellular protoplasm is consumed by normal metabolic processes. This becomes of greater significance if there is pyrexia or necrosis of tissue, as in crush injuries with anuria. Swan & Merrill (50) consider that significant hyperkalaemia may be expected in about a quarter of all cases of acute renal tubular necrosis. Death commonly occurs from cardiac arrest, but in some cases hyperkalaemic paralysis may be seen before there is serious cardiac damage (92). Measures adopted for the prevention or alleviation of this serious complication include the use of a high-calorie but protein- and electrolyte-free diet (93, 94) to reduce endogenous protein breakdown to a minimum, infusion of 40 per cent glucose into the superior vena cava together with insulin injections (95), the artificial kidney (96, 97), intestinal lavage (98, 99), peritoneal dialysis (100), and the administration of cation-exchange resins in the ammonium (101) or the sodium (102) cycle.

Hyperkalaemia may also be seen in Addisonian crisis, but it is probable that oliguria attributable to hyponatraemia and circulatory collapse is a necessary aetiological factor. Severe paralytic symptoms have been described (92, 103). Treatment consists of restoration of extracellular volume by saline infusions and DCA, together with any of the above methods for reduction of serum potassium.

¹³¹I IN THE DIAGNOSIS OF THYROID DYSFUNCTION

The use of radioactive elements in diagnosis and treatment is likely to prove of greatly increased importance as experience in their use becomes more widely available. At the moment, the assessment of thyroid function by administering radioiodine is the only application to diagnosis in internal medicine which can be considered a routine procedure. The element involved, ¹³¹I, is made by the irradiation of tellurium or, alternatively, is separated from the fission products of the pile; it is usually supplied as iodide. It has a half-life of 8.0 days and emits β - and γ -radiation, which enables it to be conveniently measured by Geiger or scintillation counters.

Since 1938, when Hertz, Roberts & Evans (104) first reported the use of isotopic iodine, a host of investigators have worked in this field, and the great number of tests which have been recommended indicates that a final

answer has not yet been found. All these tests measure the rate of some part of the iodine cycle. Administered iodide quickly mixes with the iodide present in the blood and tissues, from which it is removed almost exclusively by two organs, the thyroid and the kidney. After two to three days in normal subjects, and sometimes within 12 hr. in Graves disease (105), the thyroid has completed the concentration of the radio-element. After transformation to the hormone, the iodine is released into the circulation, enters the tissues and is later returned into the plasma as iodide. Tests have been designed to measure each step in this cycle.

Radioiodine uptake by the thyroid.—This is the most direct measurement and was the first, described by Hamilton & Soley (106, 107). In this procedure, the proportion, and sometimes the rate, of uptake in the thyroid is determined by neck counting. Frequently, measurements are made after a standard time interval (108, 109, 110); in general it is found that normal people concentrate less than 40 per cent of the dose while thyrotoxic patients concentrate more than 40 per cent in the thyroid. Unfortunately, there is an overlap between the two groups (111, 112), a limitation which is found generally in these tests.

In attempts to make the test more specific, techniques of increasing elaboration have been devised. The mathematical theory of the radioiodine cycle has proved to be a fertile field for the production of formulae and indices (113, 114). The simplest assumption, that the shape of the uptake curve can be fitted with a parabola during the earlier period, was thought at first to show a "gradient" to be calculated which was clinically useful (115, 116), but later reports do not confirm this view (117).

The most satisfying determination of function in an organ which is removing a substance from the blood is its plasma clearance. The thyroid clearance has been extensively studied and takes into account the plasma level of iodide. It, therefore, gives a result which is less influenced by kidney function and any other factors which affect the plasma iodide and, hence, the rate of uptake by the thyroid (109). As in the case of the kidney, the clearance is expressed as the volume of plasma cleared each minute and in normal people this averages 20 ml./min.

As first described, calculation of the clearance required simultaneous determination of the thyroid and blood iodine (109, 112, 118), but modified techniques have been evolved in which measurements are made over the thyroid and over a part of the body which does not concentrate iodine,—the usual site chosen being the thigh. Such measurements can be made after a standard time (105, 119, 120) or at the time of maximum thigh count when the concentration of blood radioiodide is at its maximum (121); the latter method involves more observation of the patient.

Storage of I^{131} in the thyroid and release of hormone.—Following the initial period of concentration, the iodine is slowly released into the circulation, and the content in the thyroid falls by about 0.6 per cent per day in the normal (105); in thyrotoxicosis, the rate is about ten times as great. Within a few

days, the plasma activity rises to a steady value which is maintained for a long time. It has been shown (119) that almost all this activity is attributable to protein-bound iodine, presumably as hormone.

By taking a number of measurements at intervals, the various constants of this dynamic system can be calculated, e.g., the mean duration of stay of the iodine atoms in the thyroid (105). The complications involved have made this attractive concept unsuitable for routine diagnosis. Simple measurements have been advocated, such as an assessment of the plasma activity at a standard time after a dose (122, 123, 124), with or without preliminary separation of organic and inorganic iodine.

Renal excretion of iodide.—At an early stage it was shown by Hertz and his co-workers (125, 126) that an indirect estimation of thyroid function could be made by examining the excretion of radioiodine in the urine. In principle, the thyroid and kidney compete for the available plasma iodide, and what the thyroid does not remove is excreted in the urine. As with other tests, measurement of the urine activity after a standard interval has been recommended (110, 118, 125, to 128), but this does not take into account the influence of renal function on the result. This uncertainty can be eliminated by collecting the urine over three to six successive periods (129, 130, 131), and the relation between the activities can be used as an independent check on the completeness of the collections.

The selection and use of a radioiodine test.—Most cases of thyroid dysfunction can be diagnosed on clinical grounds alone. It is only in cases of doubt that extra information is required, and several laboratory estimations can be of value. These include the basal metabolic rate, the plasma protein-bound iodine (nonradioactive), the plasma cholesterol, and one or more of the I^{131} tests. Fraser *et al.* (131) are convinced that radioiodine tests are the most useful of these.

A number of factors must be considered in the selection of a single radioiodine test for routine use. These include the dosage necessary, convenience, and time required to complete the test. The specificity of the test and the reliability of the results obtained are, of course, of primary importance, but it is evident that no single test will give all the information obtainable and that any one test will provide some ambiguous results which could possibly be resolved by testing another part of the iodine cycle.

Clearance studies by classical techniques require measurement of the radioiodine content of the thyroid, which means the use of carefully calibrated equipment to minimise the errors involved in estimating the isotope buried in the neck of a living patient. It is also necessary to take blood samples at exact intervals. In modified clearance techniques, the problem is easier because only relative counting rates need be taken; even then, relatively complicated apparatus is required to fix counter and tissue in relation to each other.

The indirect urinary estimation suffers from none of these drawbacks. The isotope is presented for counting as a solution and in an ideal form for

measurement in a liquid counter, such as that described by Veall (132). The patient and the physicist need not be together while the test is being conducted; in fact in many cases, this can be an outpatient investigation. It remains to examine the value of the results.

As mentioned above, urinary excretion tests are potentially inaccurate because they are influenced by the renal clearance. The good results reported for the simple determination of excreted iodide during a standard interval are presumably related to the normal renal function of thyrotoxic patients. The divided urinary collections described by Keating *et al.* (130) and Fraser *et al.* (129, 131) are only slightly more complicated and the "T" index calculated by Fraser has been shown (131) to be virtually independent of the renal condition. Figures 1 and 2 (published by permission of Dr. Russell Fraser) show the results obtained by several tests in patients with established diagnoses. In Figure 1, the urinary excretion of a standard period and the

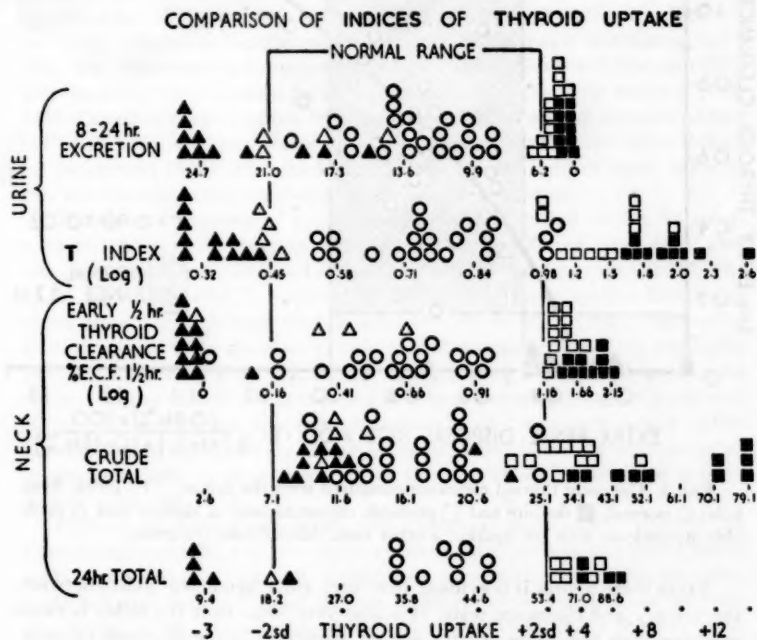


FIG. 1. The relation between certain indices of thyroid uptake including urinary excretion, net counting rates at a standard time, the "T" index, and the early thyroid clearance. The patients have been denoted by symbols according to their clinical state as follows: \circ normal; \blacksquare definite and \square probable thyrotoxicosis; \blacktriangle definite and \triangle slight myxoedema. After Fraser (In press).

"T" index are compared with neck measurements as "early thyroid clearance" [Berson (133)] and as crude counting rates at standard times. Figure 2 shows the correlation between the clearance and "T".

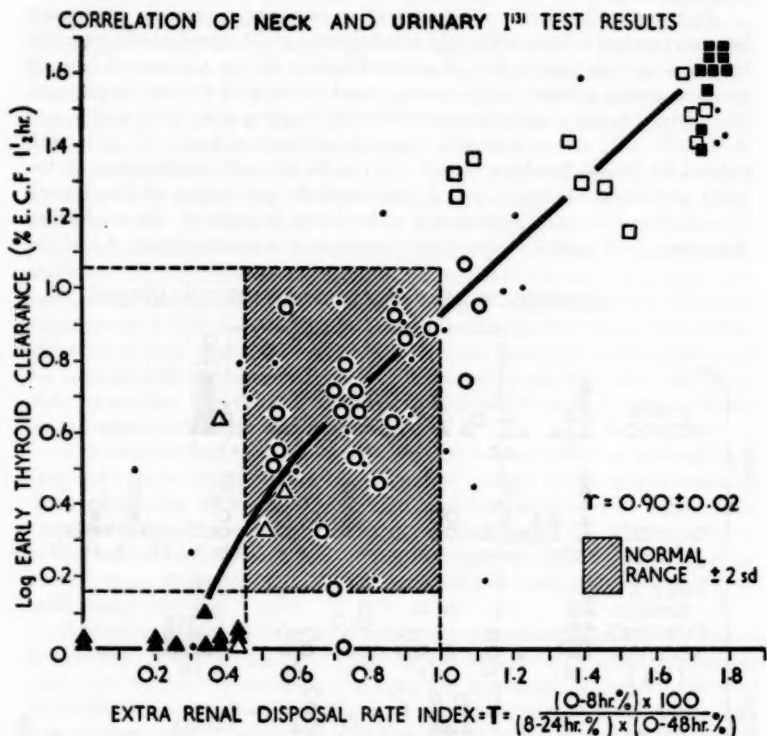


FIG. 2. The early thyroid clearance compared with the urinary "T" index. Symbols: ○ normal; ■ definite and □ probable thyrotoxicosis; ▲ definite and △ probable myxoedema with low uptake; • other tests. After Fraser (In press).

From these figures it is evident that very close agreement exists between the urinary and clearance tests. It is also very clear that the index is more sensitive than the crude urinary output, especially in the diagnosis of myxoedema. It seems to be a general feature of radioiodine tests that they are less sensitive to myxoedema than to thyrotoxicosis, and many workers have reported difficulty in distinguishing myxoedema from the normal. In this respect, a method which uses the excretion from 24 to 48 hr. seems to be especially valuable.

There is one general warning to be considered when interpreting the results of any radiiodine test. These attempt to measure the thyroid function of the patient at the time of the test and must, therefore, be related to any previous treatment which influences the activity of the gland. Such treatments include the administration of thyroxine, iodine, and antithyroid drugs, and some investigators [Foote & Maclagan (121)] do not allow their patients to eat fish, iodized salt, or root vegetables for several days before the test. On the other hand, iodine tests are objective and independent of the patients' nervousness. As with any other diagnostic test, their full value is only realized when they are considered in relation to the whole clinical picture.

AMINOACIDURIA

The development, during the last ten years, of paper chromatography of amino acids has given a great impetus to the collection of data on aminoaciduria. Previously, information of clinical value was almost confined to the identification of those amino acids, cystine, leucine, and tyrosine, which occasionally crystallize spontaneously from urine. The paper chromatography of amino acids was originally described by Consden, Gordon & Martin (134), and the early developments have been reviewed by the same workers (135, 136). The clinical applications have been described in several papers by Dent (137, 138, 139), who has recently provided an excellent account of the technical procedures (140). Microbiological assay has also been of great value in this field but must still be classed as a research procedure.

The normal excretion of amino nitrogen in the urine of adults, as measured by the formol titration method, is 100 to 400 mg. per day. This amounts to about 1 per cent to 2 per cent of the total nitrogen (137). The excretion is only slightly reduced by ingestion of a low protein diet, being much more constant than that of urea or total nitrogen (141). The amino acids in normal urine are chiefly of the so-called nonessential class, especially glycine, alanine, serine, glutamine, taurine, and β -aminoisobutyric acid (142). The last named acid is excreted in greater amounts by about 5 per cent of people, a harmless biochemical anomaly of hereditary origin (142). Urinary amino nitrogen may, however, be increased following unbalanced or inadequate diets or after ingestion or infusion of amino acids or protein digests (143, 144, 145).

Clinical conditions associated with abnormal amino acid excretion are conveniently divided into two classes: those with increase of plasma amino acids as a result of a metabolic defect, and those with normal plasma amino acids but with defective renal tubular reabsorption of amino acids from the glomerular filtrate. Concerning some types of aminoaciduria there is, however, still controversy with regard to the correct classification owing to difficulties in interpretation of small variations in plasma amino acid.

Typical examples of the former class are the aminoacidurias associated with hepatic disease and with the metabolic abnormality of phenylpyruvic amentia. Amino acid metabolism and excretion in liver disease has been re-

viewed by Dent & Walshe (146) and by Walshe (147). It is only in the most severe grades of hepatic failure, i.e., acute hepatic necrosis, that there is increase of plasma and urinary amino acids of all types; this is a result of failure of their deamination. Usually there is no obvious abnormality of amino acid metabolism in compensated hepatic cirrhosis, but in some cases there is increased excretion of cystine with or without increase of β -aminoisobutyric acid. The cystinuria is unusual in that it can only be easily detected by chromatography and not by the familiar cyanide-nitroprusside reaction (146). Using microbiological methods, Gabuzda *et al.* (148) have reported some increase of urinary methionine and tryptophan in cirrhosis.

Phenylpyruvic amentia which is a recessive hereditary defect, found in about 1 per cent of all cases of severe mental deficiency, has been shown to be attributable to a specific defect of the oxidation of phenylalanine to tyrosine (149). Phenylalanine and its metabolites, phenylpyruvic, phenyl-lactic, and phenylacetic acids (150), are present in greatly increased concentration in both blood and urine. It has been shown that a diet low in phenylalanine results in considerable clinical improvement (151).

The most common type of abnormal aminoaciduria resulting from a renal defect is that which has been long referred to as cystinuria. This condition should now be termed cystine-lysinuria to distinguish it from other conditions characterized by increased excretion of cystine. It has been shown that the cystinuria is always associated with increased excretion of lysine and in many cases of arginine and ornithine (152, 153, 154). The cystine content of plasma has been found to be normal (155). The condition appears to be a result of a specific defect of proximal tubular reabsorption of diamino acids which share a common reabsorptive mechanism (156). About 1 in 600 individuals show this metabolic defect (157), but only a small fraction of these suffer any inconvenience from the development of cystine lithiasis. Dent & Harris (158) consider that the condition is attributable either to a recessive factor or possibly to a dominant factor with incomplete penetrance.

Other types of renal aminoaciduria show a more general defect of amino acid reabsorption. In the de Toni-Fanconi syndrome there is also renal glycosuria and phosphaturia with vitamin D-resistant rickets or osteomalacia. The condition is a result of a recessive hereditary factor, and there are two distinct types of the disorder, the juvenile and the adult varieties of the disease (159). These are not genetically related, and a single family never contains both affected children and affected adults. The juvenile type (80) usually proves fatal because of associated cystinosis, i. e. deposition of cystine crystals in the reticuloendothelial system, particularly in the Kupffer cells of the liver, in the spleen, bone marrow and lymph glands, and in the histiocytes of connective tissue. It should be carefully noted that the condition of cystinosis is in no way related to the much more common metabolic defect which should now be termed cystine-lysinuria and not cystinuria. The more rare adult type (79, 160) is not complicated by cystinosis and is therefore a more benign condition although severe osteomalacia may occur from phos-

phate loss. Clay *et al.* (161) have described a specific renal lesion which is found in both the juvenile and adult types. This is a narrowing of the juxtaglomerular portion of the proximal tubule giving a so-called "swan-neck" appearance. Stowers & Dent (160) have claimed that plasma amino acids in the adult type are in normal concentration, whilst in the juvenile type Bickel *et al.* (80) consider that various plasma amino acids are in increased concentration as shown both by chromatography and microbiological assay. This would put the juvenile type of the disease into the metabolic rather than the purely renal type of aminoaciduria. It is obvious that the associated abnormality of cystinosis cannot be explained as being secondary to renal disease. In many respects, however, the renal defects of the two types show considerable similarity. Thus there may be a tendency to acidosis and also increased excretion of ketone bodies in both. This has been shown in one adult patient to be attributable to defective proximal tubular reabsorption of bicarbonate and acetoacetic acid respectively (79). Loss of potassium in the urine may lead to hypokalaemia and loss of calcium to osteodystrophy in both types. Another adult case has been described showing defective proximal tubular reabsorption of uric acid (162). It is quite clear that the defect of proximal renal tubular function in this disease has a wide spectrum and is far from being confined to a defect in relation to reabsorption of amino acids.

The aminoaciduria of hepato-lenticular degeneration (Wilson's disease) was first described by Uzman & Denny-Brown (163) and has since been confirmed by many other workers. It has been proved (164, 165) that the defect is of the renal rather than the metabolic type. Since there are interesting relationships between the amino acid defect and the more fundamental disturbance of copper metabolism in this disease, a short account of the latter will also be included. Cumings (166) has confirmed older work that there is an increased storage of copper both in the liver and basal ganglia. The specific Kayser-Fleischer ring in the cornea has been shown by histochemical methods to be attributable to the deposition of a copper compound (167). Both Cumings (168) and Denny-Brown & Porter (169) have reported considerable clinical improvement in the more chronic types of the disease in adults by injections of 2, 3-dimercapto-1-propanol (BAL) which increases the already high urinary elimination of copper. Less improvement was seen in more acute juvenile cases where irreversible cavitation of basal ganglia is often found (165). Matthews *et al.* (165) have shown that the increased urinary copper is secondary to chelation of this metal by the amino acids present in excess and that there is a close correlation between the amounts of amino nitrogen and copper actually excreted. The fundamental abnormality of the disease appears to be one of copper transport since the specific copper protein of plasma, termed " caeruloplasmin " by Holmberg & Laurell (170), is reduced in cases of the disease (171, 172). The most rational explanation of this bizarre metabolic abnormality is that all manifestations of the disease, hepatic cirrhosis, degeneration of basal ganglia, Kayser-Fleischer rings, and renal aminoaciduria are defects secondary to abnormal copper deposits in the

organs concerned. The aminoaciduria in the presence of increased tissue copper secondarily causes an increased urinary elimination of the metal by a chelation process. Attempts to produce aminoaciduria in rats by feeding excess copper (173) were unsuccessful, but other metallic intoxications in man including uranium (174) and lead (175) have been shown to cause aminoaciduria of the renal type.

Holzel *et al.* (176) have described renal aminoaciduria in association with infantile galactosaemia. Since this abnormality disappeared on a galactose-free diet, it is probable that the renal defect was secondary to the toxic effect of excess of the sugar on the renal tubules. A similar reabsorption defect has been described in the early diuretic phase of acute renal failure from lysol poisoning (177).

Increased amino acid excretion has been described during therapy of rheumatoid arthritis with ACTH and cortisone (178). A similar but considerably less effect was produced by the same means in normal subjects. Increased excretion of amino acids in association with muscular dystrophies (179) is possibly a result of excessive breakdown of muscle protein.

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MINERAL METABOLISM (ANIMAL)^{1,2}

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Since the general subject of mineral metabolism was reviewed in 1947 (1) and the trace elements in 1949 (2), several other reviews and volumes dealing with certain mineral elements have appeared. Huffman in his review of ruminant nutrition in 1953 (3) made brief reference to the relationship of calcium, phosphorus, and cobalt, and the present review will be correspondingly reduced. Copper was considered in great detail in a symposium at the Johns Hopkins University, for which McElroy & Glass (4) have edited the reports. Later, symposia on phosphorus metabolism have appeared under the same auspices, and two volumes have appeared edited by McElroy & Glass in an exhaustive treatment of this subject (5, 6). Phosphorus and its role in biology was also considered by Bertrand & Demolon (7) in a French publication. Three review articles have appeared on the general field of mineral elements in nutrition and are of particular interest to workers with laboratory and domestic animals. Green (8) has made a critical review of the effects of mineral elements in the health of ruminants. Robinson (9) has considered the trace elements and interrelationships of soils, plants, and animals while Spray & Widdowson (10) have reported on extensive studies of the composition of mammals as affected by growth and development and have reported the concentrations of many of the mineral elements. The nature of the mineral phase of bone has been exhaustively covered in a review by Neuman & Neumann (11) which has considered this important phase of mineral metabolism in animals and man. A shorter treatment of the metabolism of calcium and phosphorus in bone has been presented by Howard (12).

The restrictions of space have made it necessary for the authors of this section to limit their consideration of minerals to animals other than humans and to select critically and at times arbitrarily the subject matter to be reviewed. The availability of radioactive isotopes that can be used as tracers in the study of mineral metabolism and animals has tremendously increased the volume of literature covering various elements. In this review, isotopes are considered when used as tools in the study of the metabolism of the naturally occurring stable isotopes.

¹ The survey of the literature pertaining to this review was completed in October, 1953.

² Florida Agricultural Experiment Station, Journal Series 246.

CALCIUM AND PHOSPHORUS

Ca^{45} and P^{32} as tools in the study of calcium and phosphorus metabolism in animals have attracted many workers. Reports have appeared on the exchange rate, turnover, and distribution of these two elements in many different species. P^{32} administered orally and intravenously has been followed as secreted in the milk, and it appears that most, if not all, of the phosphorus in milk finds its origin in the inorganic phosphorus or acid-soluble fraction of the blood plasma. A comparison of the P^{32} in the plasma with that in the milk at different times after administration indicates a possibility that the phosphorus in the milk serum may come from more than one source in the blood, but if so, only a very small portion comes from the lipid phosphorus fraction (13, 14). Similar results to those obtained with radioactive phosphorus in cows were apparent in the transfer of P^{32} to the milk of goats and also in lactating bitches (31).

Reports on the distribution and metabolism of Ca^{45} in cattle have been made by a number of workers, and these reports include work on placental transfer of Ca^{45} indicating a rapid movement to the fetus from orally administered isotope (15 to 20, 24). A phosphorus deficiency occurs naturally in many areas of the world and reports continue to show the beneficial effect of adding sources of phosphorus to the diet of cattle on deficient pastures (21, 22). The close relationship between milk calcium and blood calcium is apparent in the report that interruption of milking results in a significant elevation of blood serum calcium during the period of interruption, apparently as a result of saturation of the mammary tissue (23).

The close association of calcium with the development of milk fever in dairy cattle has led to a number of studies. These emphasize the rapid drop in blood calcium, with the development of milk fever, usually within 24 hr. following parturition. Cows which develop milk fever show a severe negative balance immediately prior to parturition while normal cows are in positive balance at this time. Inorganic phosphorus follows calcium in the blood picture and shows a severe drop at the same time that serum calcium reaches a low level. Values of less than 1.0 mg. of calcium per 100 ml. of blood serum are commonly reported (25 to 30). There is also a correlation between the citric acid and calcium content of blood serum, and a marked drop occurs following calving. At parturition the drop in cows with milk fever is much greater than the drop which occurs in normal cattle.

Although it is known that calcium oxalate is not assimilated during digestion, the presence of oxalic acid does not appear to interfere with the assimilation of calcium in ruminants. Oxalates of sodium and potassium appear to be converted to bicarbonate or carbonate and if present in large quantities may induce a severe alkalosis (32).

Use of Ca^{45} in the study of calcium deposition in bones, and the metabolism of bones has demonstrated that the different bones of the body have different rates of mineral salt turnover, and within a given bone there is a

marked difference from one area to another. Ca^{45} reaches an equilibrium between bone and blood serum and tissue very rapidly, and the evidence of turnover is consistent with the concept that the bone salt exchanges calcium most readily in those portions of the bone which are in contact with the greatest circulation of blood (33 to 37). The much more rapid metabolism of the skeleton which is observed in young animals, as opposed to older animals carries over into metabolism of elements such as strontium, barium, yttrium, and cerium (38). There is considerable evidence that the body stores of calcium influence the absorption and retention of this element from the diet (39, 40).

The calcium intake just prior to measurement has a significant effect upon the subsequent utilization of calcium in the diet (40). The influence of calcium and the form of calcium on the utilization of this element and the deposition of calcium in the bone has been considered by a number of investigators in a number of different species. The guinea pig responds to excesses of calcium and phosphorus by developing abnormal deposits of calcium phosphate in the soft tissues (41). However, in other species the results are more closely associated with the formation of bone with the metaphysis best indicating the nutritional status with regards to calcium (39, 42 to 47). The upper part of the small intestine appears to be the site of both calcium absorption and calcium excretion, although both may occur to some degree in all parts of the small intestinal tract (48 to 52). Increased levels of calcium and phosphorus in the diet may induce the calcinosis syndrome with markedly increased ash content of the hearts of cotton rats (53). The metabolism of calcium and phosphorus in the fowl has been the subject of many studies with radioactive calcium and phosphorus. Absorption and distribution, and the influence of other factors on the utilization of calcium and phosphorus have been the subjects of numerous reports. The small intestine appears to be the principal site for excretion and absorption in the laying hen, and calcium is rapidly transferred to the shell with approximately 29 per cent of administered Ca^{45} recovered in the shell of the second egg (52, 54, 55, 58, 77, 78). Since the shell is the principal source of calcium for the developing chick embryo, studies have been conducted in which radioactive phosphorus and radioactive calcium have been followed from the hen through the egg into the developing chick. A maximum concentration of Ca^{45} , 1.016 per cent of the dose, was found in the chicks from eggs laid two days after the administration of the isotope. The maximum concentration of P^{32} , 0.839 per cent of the dose, occurred in chicks from eggs laid six days after administration of the isotope (56, 57). Interference with pigmentation in the New Hampshire and Rhode Island chicks has been associated with calcium deficiency and vitamin D deficiency with abnormal blackening of the feathers under these conditions (59).

The availability of dietary calcium and phosphorus is influenced by the compounds of these elements in the diet with the chick utilizing little phytin

phosphorus (60). Sulfanilamide depresses the retention of calcium and decreases the calcium in the shell (61). Interestingly enough, low calcium, phosphorus, and manganese diets adversely influence the development of the parasite *Ascaridia galli*. Smaller and fewer of these organisms occur in fowl on the deficient diets (62).

A number of papers have appeared on the effect of calcium carbonate in the diet, indicating the danger of including this form of calcium at high levels because of a depressant effect upon the other minerals (63 to 66). The level of calcium may influence the digestibility of fats adversely, particularly if these fats have a high melting point. Under these conditions both calcium and fatty acids may be unavailable in the digestive tract (67, 68).

The normal calcium, phosphorus, magnesium, sodium, and potassium values of turkey blood have been reported by two groups of workers (69, 70), and similar values have been reported for Red Sindhi-Jersey dairy cattle (71) and for sheep (72, 73). The need for calcium and phosphorus as supplemental feed sources has led to consideration of bone (74), and the bone itself as a reserve of minerals has been reported (75). An additional function of phosphorus has been suggested in the use of the artificial rumen where it has been demonstrated that phosphorus and iron stimulate cellulose digestion and urea utilization (76).

In the consideration of different sources of phosphorus for poultry and for swine, particular attention has been given to phosphatic clay or soft phosphate with colloidal clay. Work with poultry appears to be unanimous in indicating that phosphorus from this source is poorly available (79, 80, 81). A report has appeared to the effect that this product used with swine did not cause any detrimental effect when it was included in a ration already adequate in phosphorus. While not a critical test, this report indicates that the phosphatic clay may be included in the dietary of animals which are maintained for a short growing period without causing detrimental effects (82).

SODIUM AND POTASSIUM

Potassium deficiency has been described for a number of different species as well as the effects of deficiency upon various tissues within the animal bodies. Gillis (83) has shown that chicks required 0.2 per cent potassium in an otherwise adequate diet for maximum growth rate and that this level should be increased to 0.24 per cent to insure optimum performance when the phosphorus content is optimum. Diets containing inadequate amounts of potassium resulted in lowered bone calcification related to an abnormal phosphorus metabolism. Gillis repeated the chick work with rats and found essentially the same effect of lowered potassium on bone calcification. A potassium deficiency causes a lowered chloride concentration in plasma, an increased bicarbonate content, and a loss of muscle potassium with a partial replacement by sodium. The total sodium plus potassium content of the intracellular phase of muscle is lowered in potassium deficiency, and elevated

with the replacement of potassium (84). A difference was noted in the response of rats and dogs to a potassium deficiency, particularly in muscle changes, a waxy degeneration of the striated muscle fibers occurring in dogs, but not in rats (85).

In their continuing study on parturient paresis, Ward *et al.* (86) showed that cows which developed milk fever were in severe negative potassium balance while normal cattle remained essentially in equilibrium. On diets low in potassium, rats have been shown to develop low blood pressure which responds rapidly to potassium supplementation. The mechanism of this change is not known, but apparently the kidneys continue to excrete at least a minimum quantity of potassium, regardless of the potassium level of the diet or reserves in the body (87, 88). Potassium deficiency influences the composition of gastric secretion, resulting in a rise in pH and sodium content (91). Liver, kidney, and muscle reflect the deficiency by disturbance in potassium, sodium, and chloride metabolism (89 to 92, 94).

The relationship of potassium to amino acid utilization has been studied by Frost & Sandy (93), and rats depleted of protein given special diets containing a complete source of amino acids fail to respond when potassium is withheld during the repletion period. Phosphorus withheld at the same time slows recovery, but does not result in complete failure. Accumulating evidence indicates that the potassium level in the blood is regulated through the secretion of epinephrine (adrenalin) rather than by activity of adrenal cortex secretion. However, there is at least some effect of cortical hormones upon potassium absorption and excretion (95 to 98).

The potassium requirement of the chick is apparently 0.4 per cent of the diet but is affected by the rate of growth and the sodium content. If the sodium content of the diet is 0.8 per cent then a potassium level of 0.33 is adequate for maximum growth. For a slow rate of growth 0.23 per cent is satisfactory (101). These values are somewhat higher than the values of Gillis (83).

Desoxycorticosterone acetate (DCA) influences sodium metabolism, although blood pressure was not affected by the injection of DCA in dogs on a high sodium chloride intake (99). Working with DCA and potassium, Peschel *et al.* (100) presented evidence that the myocardial lesions resulting from overdosage of DCA were attributable to a deficiency of potassium rather than to the DCA. They were able to prevent heart lesions through the incorporation of a potassium salt in the diet.

Numerous reports have appeared in the literature with reference to the requirements, utilization, and toxicity of sodium chloride in the diet of various species. The chick has a somewhat higher requirement for sodium than the rat and pig (101). Sodium requirement is from 0.1 to 0.3 for the chick, depending on the growth rate, 0.05 for the rat, and 0.07 for the pig. Chlorine does not become a limiting factor in the chick with a requirement of less than 0.06 per cent if the sodium requirement is met by use of sodium

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Numerous reports have appeared in the literature with reference to the requirements, utilization, and toxicity of sodium chloride in the diet of various species. The chick has a somewhat higher requirement for sodium than the rat and pig (101). Sodium requirement is from 0.1 to 0.3 for the chick, depending on the growth rate, 0.05 for the rat, and 0.07 for the pig. Chlorine does not become a limiting factor in the chick with a requirement of less than 0.06 per cent if the sodium requirement is met by use of sodium

chloride. There is general agreement that with an adequate water supply, chicks can be fed as high as 3 per cent of sodium chloride in the diet without marked detrimental effects. Above this level, toxic symptoms appear (101 to 105). The requirements for salt appear to lie between 0.1 and 0.3 per cent, with an optimum level for laying hens of approximately 0.25 per cent.

Studies have been conducted with rats to determine their preference for diets with varying levels of sodium chloride, and it was reported that neither normal nor sodium-deficient rats have a preference for diets containing from 0.1 to 2.5 per cent sodium chloride as opposed to a low sodium diet. This is in contrast to the appetite for calcium shown by calcium-deficient rats and the avoidance of magnesium-containing diets by magnesium-deficient rats (107). The white rat has a growth requirement for sodium of 0.5 per cent, and this is independent of potassium between the limits of 0.25 and 1 per cent potassium. Potassium requirement is 0.18 per cent in the presence of 0.1 per cent of sodium, but 1 per cent sodium reduces the potassium requirement to 0.15 per cent (108, 109, 110, 114).

Results of a salt-restricted diet in the rat indicate that a deficiency of sodium chloride stimulates the adrenal cortex and its hormones influence the thymus and the spleen (111, 115). Radioactive sodium has been used in studies of sodium absorption, and it has been demonstrated that at pH 1 to 2, sodium absorption is decreased whereas above pH 11 to 12 sodium absorption is increased but is not affected between pH 2.5 and pH 10 (106). The pig apparently has a daily requirement of approximately 36 to 42 mg. of sodium, 53 to 64 mg. of chlorine, and 100 to 120 mg. of potassium for rapid growth (112). Dogs show a very different tolerance of salt from other animals because of a very efficient renal excretion which permits a normal dog to consume intakes up to 4 gm. per kg. per day for six days without an increase in body weight or in plasma sodium or chlorine (113). A report on the sodium, potassium, and chlorine content of feedstuffs has been published as an aid in the preparation of practical rations (116). In a three-year check on the preference of dairy cows for block or loose salt, it was shown that in two out of the three years more loose salt was consumed but that it appeared to be a luxury consumption (117).

MAGNESIUM

Several investigations of blood constituents as affected by parturient paresis in cattle have revealed changes from the normal magnesium values in paresis cases. Although not suggested as a causative agent, magnesium has been lower in the blood serum of cows developing milk fever than in normal animals up to 16 days prepartum. Urinary magnesium losses have been much more extensive prepartum, whereas the normal cows excreted more magnesium postpartum (28, 29, 118). Breirem and co-workers (119) have reported a relationship between magnesium and energy in the development of ketosis in cattle during their experiments under the wartime conditions of limited

feeding and particularly low magnesium pastures. It would appear that a serum value of magnesium of 2.2 to 2.4 mg. per 100 ml. can be maintained by cows receiving 20 to 25 mg. magnesium per day. Levels of 10 gm. of magnesium resulted in hypomagnesemia and combined with low energy intake, in the development of ketosis.

By following the magnesium, calcium, and phosphorus in the rumen, omasum, and abomasum of sheep through use of rumen fistulae, Garton (120) demonstrated that phosphorus and magnesium are maintained at rather constant levels in the contents of these organs, irrespective of the time of feeding or the kind of feed.

Smith (121) and Smith & Lasater (122) have investigated a magnesium potassium antagonism, and they have described an apparent inverse relationship between these two cations. Paralysis as a result of excess magnesium may be relieved by administration of potassium salts, and conversely administration of magnesium lowers the potassium content of serum rapidly. Roine *et al.* (123) have shown the importance of magnesium and potassium in the nutrition of the guinea pig with levels of 2.5 per cent potassium acetate and 0.5 per cent magnesium oxide giving normal growth on a purified ration.

Magnesium has been shown to influence protein metabolism because magnesium is necessary for the efficient utilization of amino acids in the formation of protein. Sauberlich & Baumann (124) and Menaker & Kleiner (125) have shown that with diets low in magnesium, there is excessive loss of amino acids and a failure to incorporate protein into tissue. Although the absorption of magnesium from the intestine does not appear to be affected by the presence of oxalates in the feed (126), the anion of the magnesium salt is critical in modifying the absorption of calcium (127, 128). While magnesium carbonate reduced calcium absorption, magnesium chloride, citrate, and sulfate increased both calcium absorption and urinary excretion. Another nutritional effect of magnesium was shown by Cheng *et al.* (129) who found that the absorption of higher melting fats was greatly influenced by the presence of calcium and magnesium in the diet. The removal of calcium and magnesium from the diets, for example, resulted in an increase in the digestibility of crude and refined rape seed oil to 92 and 93 per cent, respectively. Whole blood values for magnesium increased during egg production in turkeys as opposed to whole blood values of phosphorus and potassium which are decreased (130).

IRON

In contrast to the numerous reports in the literature on iron in human metabolism, the recent reports on iron in animal metabolism have been comparatively limited. In the consideration of blood formation, there have been several reviews (131, 132, 134) including some on the use of iron isotopes in the study of iron movement within the animal body (133, 135, 136). A num-

ber of significant reports have appeared on the influence of dietary factors upon the absorption and utilization of iron. Pyridoxine deficiency adversely influences iron absorption even in the presence of large amounts of iron in the diet. Work begun earlier on the anemia of infection has been continued showing that pyridoxine is essential for the absorption of iron from the intestinal tract. Other factors which influence iron absorption include protein level, a low protein diet producing an anemia and lowering the serum iron binding capacity (137, 138). It also appears that the incorporation of excess iron in the diet results in a production of a mucosal block preventing the absorption of additional iron from the intestinal tract (139, 141, 142, 147, 148). This is in contrast to saturation of the plasma with iron through injection which does not appear to control the absorption of the iron from the gastrointestinal tract (140). In a continuing study of the influence of diet on iron absorption, it has been shown that the level of phosphorus in the diet may control the absorption of iron, much of which is deposited in the liver (141, 142). Low phosphorus diets with appreciable quantities of iron result in abnormal absorption of iron from the intestinal tract and the deposition of this iron in the liver. Addition of the phosphate salts to the diet demonstrated that the quantity of iron deposited in the liver is inversely related to the phosphorus content of the diet. However, the absolute quantity of iron and of phosphorus and the iron-phosphorus ratio influence the quantity of iron absorbed. Hormonal control of iron deposition is indicated by the work of Cartwright *et al.* (143) with adrenal hormones, and by Chapman *et al.* (144), who have shown that estrogen increases and androgen decreases the liver storage of iron in pullets. McDougall (145) has shown a tremendous variation in the iron store of the livers of fetal and newborn lambs with values ranging from 21 to 600 mg. per 100 gm. of dry matter. Vosburgh & Flexner (146) working with guinea pigs and using radioactive iron demonstrated that the fetus can receive sufficient iron from maternal plasma to account for that incorporated during growth of the fetus, and that it is unnecessary to assume that the fetus derives iron from maternal red blood cells during the latter part of gestation.

COPPER AND MOLYBDENUM

The beneficial results of including copper in the dietary of ruminant animals has led to considerable research on the function of copper in metabolism of animals, both ruminant and nonruminant. An extensive symposium edited by McElroy & Glass (5) has been published covering many phases of copper metabolism in plants and animals. Likewise, Marston (149) covers many phases of copper metabolism and the interrelationships of copper with other elements. Marston (150) has proposed that copper is primarily responsible for the catalytic oxidation of the —SH group in prekeratin fiber protein to the —SS group of keratin in wool and suggests that a failure of this reaction is an explanation of the loss of crimp in the so called "steely" wool

in sheep. The Australian workers have used the rate of this reaction as a measure of the copper status of sheep.

Other workers have been concerned with the action of copper in the skin, and it appears that in formation of melanin there is an interrelationship with pantothenic acid (151 to 154). Lerner *et al.* (155) and Dawson (156) have shown that certain compounds which combine with copper inhibit tyrosinase activity, and it would appear that the action is localized in certain tissues or sites of action. Inhibition of tyrosinase would provide an explanation for the repeated observations that the most striking effect of a deficiency of copper is the loss of pigmentation in the hair coat. This has been noted in cattle, rats, rabbits, cats, and guinea pigs (151, 153, 157 to 160, 171).

Attempts have been made to ascertain the relationship of copper to abnormal bone formation, but as yet the etiology remains in doubt. Problems in practical animal husbandry include a rickets-like syndrome in calves and a fragility of bones in older animals that results from prolonged subjection to copper deficiency (150, 158, 160). The effect on bones has also been observed in foals, whereas previously it has been reported that horses appear to be immune to this deficiency symptom, ordinarily attributed to a copper deficiency in cattle (161, 162).

It has been shown that, on diets high in molybdenum with average copper intakes, the phosphorus excretion is greatly increased in cattle (163) but in rats apparently this does not occur. Bone changes have not been demonstrated in rats (164) although they have been reported in swine suffering from copper deficiency (165).

A number of investigations have demonstrated the beneficial effect of including copper in swine rations above a level that has been considered to meet requirement adequately (166, 167, 168), although no explanation of these effects has been proposed (169, 170). The close association of copper and molybdenum in ruminant nutrition has been reemphasized (149, 157, 160, 167), but other work has shown that the effect of molybdenum in monogastric species points to a much greater tolerance for this element (149, 158, 161, 172).

A series of papers has appeared on copper metabolism and the interrelationship of copper and iron in humans, swine, and other species (173 to 182). The anemia which develops as a result of copper deficiency is an iron deficiency anemia indicating that the function of copper is closely associated with (a) iron absorption by the intestine, (b) mobilization of iron from the tissues, and (c) incorporation of iron into the hemoglobin molecules.

The problem of copper toxicity has also been investigated and a few reports have recognized the hazard to farm animals and the difference in tolerance exhibited by different species. There is a difference in breeds within species as well (149). The Border-Leicester and Merino breeds appear to be much more susceptible to copper toxicity than other sheep. Cattle are quite resistant to copper toxicity (160, 183), and the greatest danger in farm

operations would appear to be those associated with grazing sheep on areas which have been heavily treated with copper (184). While it has been long recognized that molybdenum is toxic to ruminant animals and that copper in the diet affords protection from this toxicity it is only recently that a function has been proposed for molybdenum in the animal metabolism. Westerfeld & Richert (185, 186, 187), DeRenzo *et al.* (188) and Totter *et al.* (189) have demonstrated that molybdenum is a factor necessary for intestinal and milk xanthine oxidase and to that extent may serve as an essential element in animal metabolism.

MANGANESE

In a thorough going study of the relationship of the trace minerals to bovine brucellosis, workers at Wisconsin have failed to demonstrate any prophylactic or therapeutic benefit from the inclusion of cobalt, copper, iron, manganese, or zinc in the diet above nutritionally adequate levels (190, 191). However, Pottenger (192), in a report on the administration of copper, cobalt, manganese, and iodine to humans indicated that lessened pigmentation of the skin and improved chronic fatigue might result over a period of months. The information which has been developed on this subject points conclusively to the lack of beneficial effect of trace elements towards the course of brucellosis.

Bentley & Phillips (193) in their continuing study of the effects of manganese on dairy cattle conclude that while rations as low as 10 p.p.m. of manganese are adequate for growth, levels of above 20 p.p.m. are better and provide for normal reproduction. Since levels of 60 to 70 p.p.m. of manganese in the diet were adequately tolerated it would appear that a practical ration should contain manganese at levels between 20 and 70 p.p.m. Over-all performance of animals does not appear to vary greatly over a wide range of manganese intake (201), but the effect of low levels of manganese on certain tissues has been demonstrated. The actual role of manganese in bone formation is still uncertain, but it is obvious that the bones of mammals reflect the manganese content of the diet. Fore & Morton (194, 196, 197) reported that the average composition of all bone is 1.4 p.p.m. on the ash basis. Dam and his co-workers (195) show that in deficient rats the manganese of enamel and dentine is considerably higher, 0.093 as compared with 0.034 per cent in the enamel and 0.105 as compared with 0.025 per cent than in the dentine of normal rats.

The injection of manganese salts into rats and guinea pigs (198, 199) has shown the selective accumulation of manganese in the thyroid for at least a short period of time after the injection. This may have a replacing effect on iodine as suggested by Baumann & Metzger (199) who report that manganese along with chlorine, bromine, and iodine may prevent new supplies of iodine from being taken up by the thyroid with resultant thyroid hyperplasia and goiter.

Gallup and co-workers (200) have shown that manganese is excreted almost exclusively by way of the feces. In balance studies, using from 30 to 1,500 p.p.m. of manganese in the diet, they were not able to demonstrate a direct relationship between retention and level of manganese in the diet. Bernard & Demers (202) have extended the study of manganese deficiency in poultry to the duckling. Apparently ducklings react to manganese deficiency in a manner similar to the reaction of chicks. However, an optimal level of 60 p.p.m. is suggested although 15 p.p.m. prevented perosis when included in the diet. When excess manganese sulfate was included in the ration [Slinger *et al.* (203)], there was a slight depression of bone ash in turkeys. In a survey of nonbreeding cows, Bentley *et al.* (204) found that the manganese content of ovaries of these animals was definitely low, and although they could not present positive evidence as to the cause of failure to breed, they suggest that there is a possibility that trace elements are at least one cause.

COBALT

Since the last reviews (1, 2) cobalt deficiency has been reported to occur in parts of New York State. A survey of several counties (205) revealed widely scattered farms on which the hay contained 0.03 p.p.m. of cobalt or less. In general, legumes were higher in cobalt content than timothy or mixed grass hays. Lambs fed a ration consisting of hay of low cobalt content (0.02 to 0.05 p.p.m.), shelled corn (0.01 to 0.03 p.p.m.) and dried milk (0.01 p.p.m. or less) developed typical cobalt deficiency in four to seven months. The symptoms as reported earlier by other workers included decreased appetite, reduced rates of gain and then weight loss, a progressive anemia, and finally death. In addition cobalt-deficient lambs exhibited slightly lower plasma protein and calcium, and the plasma alkaline phosphatase activity was appreciably less. A simple type of anemia was typical, that is, normocytic and normochromic instead of microcytic and hypochromic as reported earlier (206). Autopsy findings were generally negative, although more than half the deficient animals showed fatty, degenerated livers. Feeding 1 mg. of cobalt daily prevented the deficiency or in deficient lambs effected recovery of appetite for the same feeds within a few days and correction of all symptoms in two to three weeks. Equal doses of injected cobalt were ineffective (207). Injection of larger amounts of cobalt at times brought about a slow, partial response in contrast to the rapid recovery after feeding (208 to 211). When radioactive cobalt was injected it passed into the rumen contents in significant amounts (212), which probably explains the slow response of cobalt-deficient animals to injection of cobalt.

With the discovery that vitamin B₁₂ contained cobalt (213, 214), the postulation was made that cobalt was synthesized into vitamin B₁₂ by rumen microorganisms and that the vitamin performed an essential metabolic function. The theory was given support by the finding that in cobalt deficiency there was a marked decrease in the numbers of bacteria in the

rumen contents and alterations in the cultural types of organisms (215). Rumen microorganisms were observed to take up selectively orally administered cobalt (216). The first tests, however, did not support the view that the physiological role of cobalt was through cobalamin (207, 208, 211), because, as was later shown, inadequate amounts of cobalamin were injected. The earlier observations that liver had therapeutic activity in cobalt-deficient animals (206) led Becker & Smith (218) and Smith *et al.* (219) to test various fractions from liver concentrates having antipernicious activity using counter current distribution procedures. It was shown that injection of certain fractions of liver extracts rapidly cured cobalt-deficient lambs, but oral administration of the same dosage level was of no therapeutic value. A re-examination revealed that when large enough doses (150 μ g. or more) of cobalamin were injected subcutaneously cobalt-deficient lambs responded favorably with increased appetite, body weight, and hemoglobin levels (219). The feeding of cobalamin had no beneficial effect. Intravenous injection of 20 μ g. of cobalamin twice daily for 21 days also was effective therapeutically (220).

The view that cobalt deficiency is primarily, if not entirely, a cobalamin deficiency is supported by the finding that rumen contents from cobalt-fed sheep are higher in cobalamin than samples from cobalt-deficient animals (217). More recently microbiological assays with *Lactobacillus leichmannii* (221) showed a cobalamin concentration in the rumen ingesta of 0.09 μ g. per gm. of dry matter from cobalt-deficient sheep compared with 1.3 μ g. per gm. for those fed cobalt. Whole blood of cobalt-fed sheep contained at least five fold more cobalamin than deficient ones. Liver concentration of total cobalamin activity was 0.06 μ g. per gm. of fresh liver from cobalt-deficient sheep and 0.93 μ g. per gm. for sheep fed cobalt with liberal rations. Vitamin B₁₂ (cyanocobalamin) and B_{12b} (hydroxycobalamin) appear to have approximately equal activity when injected into cobalt-deficient sheep (222).

Recent evidence (223) suggests that even in rats and mice there may be limited utilization of cobalt for synthesis of cobalamin probably by intestinal microflora. This is in contrast to earlier failures to demonstrate a deficiency of cobalt in the diet of rats and rabbits (1, 2). The addition of cobalt to diets composed of corn and soybean oil meal and deficient in cobalamin has resulted in growth stimulation of chicks (224), but not when cobalamin was also added to the diet.

Additional studies have been made on the distribution of radioactive cobalt in animals (225, 227). Prolonged feeding of relatively large doses of radioactive cobalt resulted in far greater tissue concentrations of "activity" than previously observed (226). Largest amounts were found in the liver, heart, kidney, and pancreas, although muscle equaled liver in total cobalt storage, because of its greater mass.

Oral doses up to 40 to 50 mg. of cobalt per 100 pounds of body weight were tolerated by cattle without ill effects (228, 229). Larger intakes resulted in reduced feed intakes, loss of body weight and increased hemoglobin, and

packed red cell volume of the blood. No difference was found in the toxicity of the chloride, sulfate, or carbonate of cobalt. With sheep the tolerance was up to 160 mg. of cobalt per 100 pounds of body weight for oral administration, but 75 mg. per day by intravenous injection caused death (230). No polycythemia occurred with sheep. The lethal dose of cobalt injected intravenously for the dairy calf was reported to be more than 4 mg. per pound of body weight (231). Symptoms of toxicity included lacrimation, salivation, dyspnea, and incoordination. Injection of methionine previous to cobalt injection prevented or greatly reduced the severity of the toxicity symptoms.

SULFUR

A revived interest has been observed in the use of inorganic sulfur compounds by ruminants since the direct demonstration (232) that urea nitrogen and inorganic sulfates permitted synthesis in sheep of the 10 amino acids essential in the diet of the rat. When sheep were fed a sulfur-deficient diet (233) they went into negative balance for sulfur and nitrogen, lost weight and eventually died. Pair mates receiving calcium sulfate stored both nitrogen and sulfur. Wool growth was retarded in the sulfur deficient animals but its sulfur, nitrogen, or essential amino acid content was not altered. This study suggested that inorganic sulfur can probably suffice for the formation of all the sulfur-containing compounds of the body because of microbial synthesis in the gastrointestinal tract. Elemental sulfur also has been shown to be utilized by growing lambs (234).

Radioactive sulfate was incorporated into the methionine and cystine of milk of cows (235) and goats (236) as soon as three hours following ingestion of S^{35} . Tagged methionine and cystine were isolated from bacterial protein of the ruminal contents. In the hen, radioactive cystine was isolated from albumen and yolk of eggs following injection of S^{35} , but the methionine contained no activity (237). Following oral doses of S^{35} the blood and liver of sheep contained five to eight times the activity of similar tissues from the rabbit suggesting a higher level of microbial synthesis in the gastrointestinal tract of the sheep (238).

In view of the above results, it is interesting that the addition of sodium sulfate or of methionine to sulfur-low rations (0.1 per cent sulfur) for lactating cows did not increase milk production or the efficiency of feed utilization (239). Data on quantitative requirements of sulfur are not available.

FLUORINE

The problem of fluorine toxicity in animals has been recently reviewed (240, 241). The existence of several problem areas in the United States has stimulated new research to define more accurately the safe tolerance levels for cattle over long periods of time. Hobbs *et al.* (242) added sodium fluoride to give rations containing various levels up to 100 p.p.m. of the air dry feed. Beef cows showed mottling and erosion of the teeth on rations containing

40 to 50 p.p.m. of fluorine or more. Careful studies of animals grazing fluorine-toxic areas or consuming water high in fluorine have again confirmed the symptoms previously reported, that is, mottled enamel of the teeth, excessive erosion, rough hair coat, tight skin, unthrifty appearance, and exostoses and enlargements of the bones (243, 244). The extent to which diarrhea, low production, poor condition, and general unthriftiness may be the result of mild fluorine toxicity in borderline areas, or from other causes, is sometimes difficult to determine with certainty (245). The possibility has been mentioned (246) that the use of corn distiller's dried solubles, which has been shown to contain 24 to 244 p.p.m. of fluorine, may at times contribute to the fluorine problem in animals. The problem is critical primarily during growth of teeth and bones.

Attempts to elucidate the mechanism of fluoride action have given some interesting leads, but a final answer has not been presented (247). By the use of autoradiograms of bones of pigs receiving Ca^{45} , it was shown that fluorine feeding results in "lack of definition at the position of original epiphyseal laydown" compared with sharp demarkation for normal animals (248).

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THYROID HORMONES AND IODINE METABOLISM^{1,2}

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This review covers the most important problems concerned with thyroid hormones. Its scope does not include all phases of iodine metabolism. Papers of recent symposia (1) or general surveys (2 to 8) are a suitable source of documentation on the subject. Thus, this review will deal chiefly with the biochemistry of iodine in animal organisms and be devoted to the formation, secretion, and metabolism of iodine. As some of these subjects are directly related to chemical processes of protein iodination, it seemed necessary to consider progress with artificial iodoproteins.

Important advances have been achieved in the past on specific points of iodine biochemistry, but they have remained unconnected. The current line of research is to orient coordinated studies along certain paths. On one hand, considering that the iodination of amino acids and the formation of thyroid hormones take place in a protein molecule, the biochemistry of the natural iodinated derivatives has been usefully connected with that of protein halogenation. On the other hand, successive steps in the process leading to the formation of biologically active thyronine derivatives have been characterized and their sequence established; thus the study of the mechanism of each has been rendered possible. Finally large uses of radioautographic (9, 10) and radiochromatographic (11) techniques led to the isolation of new derivatives and rendered possible metabolic studies of minute amounts of marked iodinated compounds under physiological conditions. Thus, new orientation of research and important technical progress have permitted renewed study of many problems.

IODINATED AMINO ACIDS AND PROTEINS

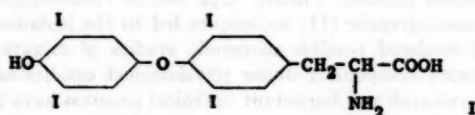
Amino acids and thyroid hormones.—Until 1947 L-3,5-diiodotyrosine (DIT) and L-thyroxine, i.e., 3,5,3',5'-tetraiodothyronine (Tx) (see Formula I), were only known natural iodinated amino acids. L-3-monoiodotyrosine (MIT) prepared by synthesis (12), has been found recently to be a constituent of thyroglobulin (11, 13, 14) and, in minute amounts, in thyroid gland extracts as free amino acid (15). It is also present in some scleroproteins of invertebrates, the gorgonins, and spongins (16), with 3-mono-(17) and 3,5-dibromotyrosine (18, 19, 20), and in the artificially iodinated proteins (21, 22, 23).

¹ The survey of the literature pertaining to this review was completed in July, 1953.

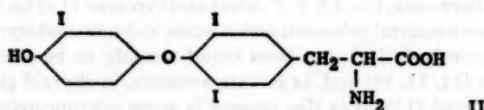
² The following abbreviations are used in this chapter: ATP for adenosinotriphosphate; DIH for 2,4-diiodohistidine; DIT for L-3,5-diiodotyrosine; MIH for monohalogen derivative of 2(or 4)-diiodohistidine, MIT for L-3-monoiodotyrosine; TrITH for L-3,5,3'-triiodothyronine; TSH for thyrotropic hormone; Tx for 3,5,3',5'-tetraiodothyronine.

Ten to fifteen per cent of total I of thyroglobulin are included in MIT, but as the iodine content of this is usually lower than 1 per cent, the maximal percentage of MIT is 0.1 per cent to 0.2 per cent; it can reach up to 5 to 7 per cent in gorgonins, and some of them contain up to 10 per cent I. DIT, isolated from all animal iodoproteins, has been found in hydrolysates of marine algae (24). Iodohistidines also have been detected in iodoproteins (25, 26) a few years after their synthesis (27, 28). 2-4-Diiodohistidine (DIH) has been found only in artificially iodinated globin or casein (29), but a monohalogen derivative (2 or 4 MIH) is present in these as in thyroglobulin (26) and in thyroid extracts (26). It includes 1 to 3 per cent of total I in the gland. All these derivatives do not show the physiological activities of the thyroid hormones.

The most important advance in this field is the simultaneous isolation in 1952 of a new thyronine derivative, the L-3,5,3'-triiodothyronine (TrIth) (see Formula II) from enzymic hydrolysates of thyroglobulin (30, 31) and the preparation of it by iodination of L-3, 5-diiodothyronine (32) by two groups of biochemists working independently. TrIth and Tx are the only natural amino acids containing the thyronine nucleus isolated in a pure state until now (33, 34, 35). Unsubstituted thyronine has never been detected in a protein, and its formation seems to be bound to oxidation processes involved by iodination. Traces of diiodothyronine (3,5 or 3,3') are possibly present in thyroglobulin.



Thyroxine or 3,5,3',5'-tetraiodothyronine (Tx)



3,5,3'-triiodothyronine (TrIth)

The natural L-TrIth has to be considered as a thyroid hormone, just as thyroxine. Both are formed and secreted in the gland and are present as free amino acids in gland extracts (36) and are constituents of artificially iodinated casein (37). Physiological activities of L-TrIth are distinctly higher than those of L-Tx, 5 times more on respiratory exchange of myxoedematous patients (38) or of rats (39, 40). TrIth decreases the uptake of I^{131} by the

thyroid gland when doses of 10 times less than Tx are given (41). TrIth (1 mg. daily) prevented pituitary enlargement in thyroidectomized rats (42). Thyroxamine or 3,5,3'-triiodothyronamine may be the epinephrine sensitizing substance produced by intestinal mucosa (43, 44); the first has been tentatively characterized by paper chromatography (45).

The synthesis of all these substances labelled with I^{131} has been worked out in order to study their metabolism in minute amount of physiological order of magnitude. Chromatography on paper allowed the preparation of small quantities of MIT, DIT, TrIth, and Tx obtained by direct iodination of amino acids (28, 46). Tx labelled on 3,5 or 3'5' or 3,5,3',5' and TRITH labelled on 3,5 on 3' and on 3,5,3' have been synthesized with high specific activities by special techniques (33, 35, 47) and Tx with C^{14} in the alanine residue (48) also has been synthesized.

Natural and artificial iodoproteins.—Two types of iodoproteins have been known for a long time in animal organisms: thyroglobulin of the thyroid gland of vertebrates and scleroproteins of supporting tissues (corneous skeleton) of various invertebrates, gorgonins of sea-fans and spongin of sponges; they have been newly studied. Small amounts of iodoproteins appear to be present in the egg yolk of birds (49, 50, 51) and in some seaweeds (24), because enzymic hydrolysis of both liberates iodotyrosines (DIT² and, in the first case, MIT²). Administration of labelled iodides to chicken or immersion of *Laminaria digitata* in sea water to which NaI^{131} has been added showed this fixation. In egg yolk the fixation of labelled iodine is much lower than in thyroid gland, and it proceeds at a lower rate. The maximal organically bound iodine is present in 6 to 8 days after injection, and the transformation of iodide ions into iodoproteins takes a few days (49).

Thyroglobulin has been studied as a protein and as a source of iodinated amino acids formed by the action of the halogen on the initially halogen-free molecule. Its labelling with I^{131} in the gland of animals injected with tracer doses of NaI^{131} or in gland slices immersed in an isotonic fluid containing the same salt gave new facilities to research on its purification and properties (52). The only peculiar feature of this globulin in its amino acid composition is its high percentage of arginine [arginine = 12.4 per cent (53)] (See Table 1). Some of the peptide bonds in which arginine residues take part are of an exceptional stability towards acid hydrolysis compared to the arginine peptide bonds in other proteins (54). The iodinated amino acids are not preferentially liberated by chemical or enzymic hydrolysis, and none of the pure pancreatic proteinases show specificity for it (55). Small differences in cystine and tryptophan content have been found between thyroglobulins of different mammals (56), but not systematically between these of human normal and pathological organs (52). Thyroglobulin of glands of dogs or guinea-pigs treated with 6-propylthiouracil as goitrogenic agent shows an abnormal behavior in its solubility (57) and electrophoretic migration (58), and that of very enlarged goitrogenous gland of pigs or calves, is poor in cystine (59). Previous data on molecular weight ($M = 600,000$) and pH (4.6) have been confirmed on high-

TABLE I
AMINO ACID COMPOSITION OF HOG THYROGLOBULIN

Amino Acid	Per Cent in the Protein	Amino Acid	Per Cent in the Protein
Arginine.....	12.72	Cystine.....	3.60
Histidine.....	2.23	Methionine.....	1.30
Lysine.....	3.42	Alanine.....	4.40
Phenylalanine.....	6.68	Glycine.....	3.70
Tryptophan.....	2.08	Leucine.....	12.80
Tyrosine.....	3.12	Valine.....	1.45
Diiodotyrosine*.....	0.54	Serine.....	10.80
Thyroxine*.....	0.21		

* The thyroxine and diiodotyrosine levels, as well as the concentration of total iodine 0.48 per cent, do not have absolute significance; they vary from one preparation to the next.

ly purified preparations, electrophoretically homogeneous (53, 60). These contained three fractions of slightly different solubility, but of the same I/N ratio (56). The iodine content is not the same in various equally pure preparations from different origins; values from 0.1 to 1.23 per cent have been found, the highest in proteins from foetus of calves (61). The differences in iodine content are related to the efficiency of iodination mechanism in the epithelial cells of the gland. These cells produce an unhalogenated protein of constant amino acid composition and structure as a substrate for iodination. This process is autonomous, and its efficiency is governed by biological factors; it can thus lead to a higher or lower level of iodination of the protein. Interference between protein structure and physiological function of thyroglobulin remains unclear. The only argument for its importance is that, whatever the iodine content is, the ratio: iodothyronines I/total I is of about 0.3. It shows that the ratio of iodotyrosines to iodothyronines is nearly constant, but this may be attributable more to identity of halogenation conditions than to the nature of the protein (59).

The amino acid composition of scleroproteins of invertebrates containing up to 9 to 10 per cent I, and usually much less Br (62), has been extensively studied (63). Only traces of Tx are present. L-MIT and L-DIT are the chief halogenated amino acids, with some of their brominated isologues. The reason for this difference with thyroglobulin appears to be a structural one. In the rigid scleroprotein fibers, most of the tyrosine residues are blocked in position preventing their joining to form thyronine, as in silk fibroin (64) which is unable to produce thyroxine by iodination. The composition of the scleroproteins gorgonins and spongins is more or less characteristic of zoological species. They permit in such cases as the sea-fans of the genus *Rhipidogorgia* a fairly good control of zoological classification. In the same field, it has

been shown that the proteins of the horny skeleton of a group of Hexacorallia, the *Antipathidae*, are very different from the gorgonins of Octocorallia, with which they were confused. They are a new type of scleroproteins, called antipathins, characterized by a very high histidine content (up to 17 per cent). The amino acid composition of antipathins shows specific differences, but their histidine content has never yet been under 12 per cent, instead of 2.5 per cent in gorgonins. The zoological position of the *Gerardiidae* (black coral of Red Sea district) as Hexacorallia has been discussed from these biochemical findings (65). The comparative composition of spongins from various sponges has been also studied, especially their iodinated and brominated constituents (66); these proteins show specific differences.

Chemical iodination of proteins has been extensively studied in the last ten years, not only for preparation of large amounts of substitutes of thyroid gland preparations, but also as "models" of physiological thyroxinogenesis. Only recent papers will be referred to here, as a suitable review of previous work (5) is available.

It has been known since 1937 that Tx is formed by iodine action on proteins (67). It has been recently shown that TrIth is liberated from these by alkaline hydrolysis (37). As deiodination is fairly severe during this process, (68), it was necessary to control the presence of TrIth in safer experiments, as had previously been the case in the detection of MIT. This has been done by enzymic hydrolysis, conducted without any I⁻ ion formation (69). The significance of this for the understanding of the mechanism of hormone formation will be discussed below. Research on thyroxinogenesis by chemical means deals with the production of a mixture of Tx and TrIth or of Tx only according to the iodination conditions. The two thyronine derivatives have very similar analytical properties (solubility in *n*-butanol at various pH levels, color reactions) and have, therefore, been determined nearly always simultaneously (41). This does not create severe difficulty in interpreting the results, because the chief interest of the research is the study of thyronine formation, whether tri- or tetrasubstituted.

Tyrosine residues of proteins are substituted in 3-(MIT) by small amounts of I (maximal MIT content with 2 to 3 atoms I per mole tyrosine) and in 3,5-(DIT) with larger amounts. Therefore, Tx is present only if an excess of halogen is used under suitable conditions (pH, temperature, reaction time) (70). Tx yield under identical treatment, depends on the nature of the protein. The factor governing the yield is not the tyrosine content, but the structure of the protein. Thyroglobulin is not especially suited to iodination; the maximal production of Tx per tyrosine residue has been reached with casein and insulin (71), the minimal with silk fibroin (dissolved) (64). It is not possible to control exactly the percentage of tyrosine able to be transformed in Tx; a part of this may be destroyed by oxidation. The value of 1.5 to 2.0 per cent in iodocaseins is probably a maximum. Incubation (pH = 7.5, 70°C, 72 hr.) of N-acetyldiiodotyrosylgelatin leads to the condensation of about 10 per cent of DIT residues in Tx (72).

CHEMICAL MECHANISMS OF IODINATION OF PROTEINS AND
FORMATION OF IODOTHYRONINES

Iodine acts on proteins as a substitution and oxidation agent. The total amount of reagent saturating a protein by substitution on cyclic amino acids is proportional to the tyrosine and histidine contents. Phenylalanine is not substituted and iodotryptophan formation probably does not take place. Substitution on nitrogen atoms ($\cdot\text{NH}_2$ or $:\text{NH}$) does not occur in nature and N-iodinated derivatives are unstable in neutral or slightly alkaline media.

Tyrosine is substituted in position 3 (MIT) and 3,5. (DIT) in proteins before histidine, on which I fixes in 2 (or 4) as MIH^2 , in 2 and 4 as DIH , except in histidine-rich proteins, like globins, where MIH can form simultaneously with DIT.

It has been admitted until recently that only disubstituted derivatives of both amino acids appear during halogenation of amino acids. This scheme was apparently in agreement with kinetic studies (73 to 76). Research based on the chromatographic separation of the products of the reaction with increasing amounts of the halogen rendered it possible to follow the two successive reactions: (a) $\text{RH}_2 + \text{I}_2 = \text{RHI} + \text{HI}$ and (b) $\text{RHI} + \text{I}_2 = \text{RI}_2 + \text{HI}$. When less than 3 atoms I react with 1 molecule of free tyrosine or histidine, RIH is the main product and RI_2 only when 6 to 8 atoms I react. In the case of tyrosine the substitution reaction proceeds without oxidation. In that of histidine, it breaks the imidazole ring (26). Tryptophan treated in the same way is not substituted, but oxidation destroys the indole ring (77). The same reaction has been applied to the phenolic ring of 3,5-diiodothyronine; it produces successively TriTh and Tx (33). Bromination of amino acids proceeds in the same way as their iodination (78). Then, the formation of mono- and disubstituted halogen derivatives of the cycles of tyrosine, histidine, and 3,5, dihalogenothyronine are successive chemical steps of the substitution of these amino acids. It proceeds identically with proteins, leading to mono- or dihalogen derivatives according to the amount of halogen reacting.

Iodine is predominantly an oxidizing agent in some circumstances, not only in opening nitrogen-containing imidazole or indole rings, but also, apparently, in promoting the formation of iodothyronines (Tx and TriTh). In proteins, substitution alone takes place with relatively small amounts of iodine. With 4 to 6 atoms I per tyrosine residue and under proper conditions (79), a condensation reaction produces TriTh and Tx from tyrosine residues. A more or less maximal yield of Tx is reached by increasing the iodine amount in a slightly oxidizing medium (NH_4OH , 37°C .) and an optimal yield for 6 to 8 atoms I when oxidation proceeds (incubation at 70°C ., with $\text{Na}_2\text{CO}_3 + \text{NaHCO}_3$, $\text{pH} = 8.0$) (80). The mechanism of the reaction is not well known, although it appears to be of an oxidative type, catalyzed by free iodine (81). Neutral or slightly alkaline DIT solutions incubated at 37°C . contain after a few weeks traces of Tx (82). The yield of the reaction can at-

tain 4 per cent in a few hours if H_2O_2 is added and Tx is extracted continuously on a boiling water bath (81). It is increased if the amino acid is replaced by its N-acetyl derivative (yield 7 to 10 per cent) (83) or, better, by some of its peptides. With N-acetyldiiodotyrosylglutamic acid, it reaches 35 per cent (84) and shows a very definite optimal pH (7.5). Incubation of various tyrosylpeptides with iodine led to the conclusion that the state of $-COOH$ or $-NH_2$ of the alanine chain of tyrosine has probably no influence on the condensation reaction; 32 per cent Tx is obtained from leucyltyrosine (85). Derivatives of the alanine residue eliminated by the condensation into thyronine of 2 moles DIT have been characterized. They include oxidation products, such as pyruvic acid (86), serine (87), and also N-acetylalanine in the case of N-acetyl DIT (88). Therefore, some authors consider that the reaction involves the formation of a labile intermediary of quinonic structure (86, 89), liberating dehydroalanine which is transformed later into pyruvic acid (oxidation) or into serine hydration; others admit a single dismutation. The chief argument supporting the first opinion is that oxidizing agents such as H_2O_2 favor Tx formation. I_2 is probably responsible for the apparently spontaneous condensation in DIT solutions (81, 82), as a significant amount of iodide is always found in the incubation media. The mechanism of the reaction is probably the same for DIT, its peptides and proteins and it seems also to apply to TrITH formation. As this is found in iodoproteins in conditions where its origin from deiodination of Tx is highly improbable, it can be considered that condensation of MIT and DIT leads to TrITH just as the one of 2 molecules of DIT leads to Tx (34). Thus the results of studies on amino acid and protein halogenation allow a better understanding of the basic chemical processes of thyroid activity. In the actual state of our knowledge in this field, no difference has been found between the two; therefore the chemical model is of very great interest for new biochemical approaches.

BIOSYNTHESIS OF THYROID HORMONES

Iodine metabolism starts from iodide ions, always present at a very low level in body fluids or in surrounding media of aquatic organisms (0.5-1 $\mu g.$ per 100 ml. of plasma, 1 to 4 $\mu g.$ per 100 ml. of sea water). A powerful concentration procedure must, therefore, exist in order to collect these ions and allow iodine to enter into metabolic cycles. It is the most efficient in the thyroid gland of vertebrates.

The mechanism of concentration of iodides by the thyroid is its most specific function. Location of I^{131} in various regions of epithelial cells of thyroid gland and its integration into thyroglobulin has been followed by autography (90). Normally it is able to assure the concentration of about 10 $\mu g.$ per 100 gm. of tissue while the blood level is less than 1 $\mu g.$ per 100 cc. (91, 92, 93). If iodine fixation on thyroglobulin is prevented by thiouracil the ratio thyroid I/blood I^- is close to 25 (94). This ratio can greatly vary. It increases after action of the thyrotropic hormone (95) and in Graves' dis-

ease (94, 96). This concentrating ability is greatly lowered by the presence of large amounts of iodide (97, 98), by continued administration of thyroxine (99), and by hypophysectomy (100).

Iodide ions probably penetrate into the bordering cells rather than into the lumen of the follicle, while the similar concentration in other organs results in an increase of iodide in the secretions, as in the salivary glands and in the stomach (101).

Recent data have been added in this field by work on the thyroid gland of fishes (102 to 106) and on the comparative efficiency of maternal and foetal gland in pregnant cows (62). Concentration is also effected by the ovary of mammals (107) and by the egg yolk of chicken (49). It is easy to follow in marine sea weeds of *Laminaria* immersed in sea water containing I^{131} (24). It was shown by autoradiography that initial trapping of I^- by algae takes place in an apparently specialized island of cells. The method of collecting iodide by living cells is not known. It probably does not involve the oxidation to I_2 necessary in the subsequent biological step, because it is possible to find, in thyroid gland of animals treated with thiouracil derivatives, "loosely bound iodides" freed as I^- by a single trichloroacetic acid treatment (108). Maximal amount of free iodide in the gland is nearly 1 per cent of total I in normal animals (109). Br^- is much less efficiently concentrated and remains only a few hours in the gland where it takes no part in substitution reactions on tyrosine (110, 111, 112). It is likely that oxidation of I^- into I_2 (or IO^-) is an independent step in iodine metabolism, of enzymic character (113). However, one cannot exclude entirely the possibility that the step occurs concurrently with concentration of the same ions in normal organs, because substitution reaction will restore I^- from H. It seems that tissue integrity is necessary to the trapping of I^- (114); this may depend on general physiological factors of selective permeability of the cells, although experiments were performed with homogenates (115). Concentration is controlled by two pituitary systems (116) probably corresponding to two TSH (thyrotropic hormone) one of which would regulate the iodide trapping mechanism, the other producing a glandular hyperplasia. There might, however, exist a regulation of the iodine pick-up which, independent of pituitary secretion, would be in direct relation with the amount of iodine present in the gland (99). Oxidation is no doubt an enzymatic process. It is probably attributable to cytochrome oxidase in organ slices (117, 118), but may also involve a peroxidase (119, 120) and a xanthine oxidase (121, 122). Br^- is not oxidized by these enzymes in the gland, which explains why bromotyrosines are not formed like iodotyrosines in thyroglobulin of animals treated with bromides. However, Br^- must be a substrate for oxidases of invertebrates involved with bromination of gorgonins and spongins, probably acting on much higher potentials. This step of iodine metabolism requires further investigations to clear its evolution, but the formation of I_2 is chemically necessary for hormonogenesis. The complete and rapid oxidation of thiourea with production of SO_4^{2-} in the gland (123) shows it indirectly.

The biological fate of I_2 is bound to its reactions with thyroglobulin. The first studies of Chaikoff and his colleagues (124) on the sequence of formation of "diiodotyrosinlike" (DIT and MIT) and "thyroxinlike iodine" (Tx and TrIth) led firmly to the conclusion that, as admitted before by Harrington from chemical evidence, DIT is the precursor of Tx. The isolation of MIT and TrIth made it necessary to define more precisely the biosynthesis of thyroid hormones; this has been possible using radiochromatography for the analysis of labelled thyroglobulins prepared from animals killed at successive time periods after injection of I^{131} . MIT is regularly formed very rapidly (34); it is found in relatively large amounts in 2 hr., and its level reaches a maximum in 8 to 10 hours and decreases later, but remains surprisingly high at least 48 hr. (125) for various reasons. The level of DIT increases less rapidly and shows a maximum a few hours after MIT. Labelled Tx and TrIth appear after a longer delay. TrIth predominates at the eighth hour and Tx after, the maximal level of the first is reached within 12 to 14 hr., that of the second in 24. Thus, the situation appears more complicated than predicted by the initial scheme, except that the iodotyrosines must, beyond discussion, be considered as the precursors of the iodothyronines. The MIT maintained at a high level and the origin of TrIth had to be explained.

Both derivatives could be bound to partial deiodination of more halogenated substances; therefore biological dehalogenation had to be studied. Orientation work on enzymic deiodination of DIT by various organs has been done (126), one of them (127) with better technical control than the other, has been performed simultaneously with research on labelled substrates on a much wider field and including radiochromatographic control of the products of the reaction (128). Slices of thyroid gland of sheep immersed in an isotonic solution containing DIT liberate MIT and I^- , before deiodinating MIT. Its formation excludes the interference of a transaminase action leading to 3,5-diiodo-4-hydroxyphenyl pyruvic acid, a substance deiodinating spontaneously in solution (126). Dehalogenation by $-SH$ groups, as it takes place in hepatic extracts containing proteins in the presence of aliphatic chloro-derivatives (129) is also excluded; this reaction is not extended to cyclic isologues. The dehalogenation is much more energetic with thyroid slices than with liver, intestine, or kidney; it does not take place with those of other tissues. The enzyme responsible called "dehalogenase," has been extracted from cell-free solutions and its character studied (130). At optimum pH 6.8 to 7.0 it decomposes specifically MIT, DIT, and their brominated isologues, but none of the iodinated or brominated derivatives of thyronine studied (Tx, TrIth, DITH and the brominated homologues), nor thyroglobulin.

The physiological role of this enzyme appears to be very important in controlling the selectivity of the secretion of TrIth and Tx, as will be discussed below. The continuous deiodination of MIT and DIT has an immediate consequence for the I^- supply of the gland, as established by the study of

thyroglobulin of slices of thyroid tissue immersed in thoroughly oxygenated isotonic liquid containing labelled DIT. The iodides freed from this are recovered by the epithelial cells of the gland and enter again into the iodination cycle of the protein (131). Enzymic hydrolysis liberates the series of labelled amino acids formed from endogenous iodine (132). As proteolysis of thyroglobulin is continuous in the gland, dehalogenation of MIT, DIT, and secondarily MIH provides a continuous source of I^- for the organ, which explains the prolonged high level of labelled MIT after administration of tracer doses of I^{131} .

Two possibilities might explain the origin of TrIth. It could be a deiodination derivative of Tx (35) or a primitive product of condensation of MIT and DIT (34). The inefficiency of thyroid slices and of purified dehalogenase on all thyronine derivatives is against the first hypothesis; on the contrary, a few arguments support the second. TrIth appears very early after administration of I^{131} , and its concentration in the gland decreases when that of Tx increases (34). The formation of TrIth into thyroglobulin, in which deiodination appears not to take place, can be interpreted as a condensation process of 1 mole MIT with 1 of DIT. This is not surprising if it is kept in mind that MIT is continuously formed on a fairly high level as a result of the endogenous production of I^- . If so, TrIth originates by the same mechanism as Tx; the only difference resides in the initial material of the condensation reaction. But, the substitution reaction in the epithelial cells cannot choose between tyrosine and thyronine cycles, and it proceeds continuously too. Therefore, a part of TrIth can not escape iodination on carbon 5' and is transformed into Tx. Thus, MIT is a precursor of both thyroid hormones and TrIth a precursor of Tx much more than its derivative.

It is important to point out again that TrIth is a constituent of thyroglobulin just as Tx and that they appear as amino acids taking part in the structure of this protein. As both substances have qualitatively identical properties, it is logical to consider both as hormones produced and secreted by the gland. Even if Tx is transformed into TrIth by the cells of tissue, the initial hypothesis that TrIth is the only physiological hormone is too restrictive.

SECRETION, CIRCULATION, AND METABOLISM OF HORMONES

Chemical mechanism of secretion and circulating hormones.—As Tx and TrIth are incorporated in the peptide chains of a protein, these must be hydrolyzed. None of the proteases tried (pure pancreatic proteases, papain, catheptases) preferentially free the iodinated amino acids of thyroglobulin, nor show a clear specificity for peptide bonds of the hormones (55). Two catheptases (pH optima: 3.5 and 6.5, activation by KCN and —SH) have been detected in the gland (133, 134, 135). One is adsorbed on thyroglobulin in saline extract of the organ and has been purified (136). As liver catheptase is activated by Tx (137), it may be that the thyroid enzyme behaves similarly. All iodinated amino acids of thyroglobulin have been found in butanol extracts of the gland as labelled substances, identified by autography of chro-

matograms, 24 hr. after injection of I^{131} to rats (15). MIT, DIT, TrIth, Tx and MIH are present with small amounts of unknown derivatives, probably peptides, comprising altogether roughly 1 to 2 per cent of the total iodine of the gland. Proteolysis is not only a necessary step in hormone secretion, but it renders this easier by increasing osmotic pressure in organ fluids. But another phenomenon interferes to allow a sort of physiological choice between iodinated amino acids of the gland extracts and leading to the selective diffusion of the iodinated amino acids. The circulating hormone must be studied before describing this mechanism.

Tx and TrIth are present in blood plasma. They can be extracted from it (138 to 141) by butanol and identified by paper chromatography (radioautography). Both precipitate with plasma proteins on treatment by many precipitating agents, migrate electrophoretically with the α_1 globulin (142), and do not dialyze in water (138). They are not chemically combined to these proteins, but absorbed on these; Tx added to serum behaves in this respect exactly like the one circulating in blood (31). The percentage of Tx and TrIth iodine in plasma of mammals is from 60 to 80 per cent of the organically bound iodine and that of iodide ions 10 to 20 per cent. Tx largely predominates over TrIth, as in thyroglobulin or in thyroid extracts; its level is 3 to 4 times that of TrIth. The origin of the latter one in plasma has been discussed (125). It was considered at first that TrIth could be a deiodination product of Tx diffusing from tissue cells during metabolic utilization of the latter and possibly not secreted by the thyroid gland. As the injection of tracer doses of labelled Tx is not followed by the appearance of labelled TrIth in blood, it is probably not necessary to retain this opinion. Very minute amounts of other organic iodinated derivatives are also present (143), particularly MIT and DIT in noticeable traces, by autography of chromatograms of I^{131} labelled spots. In rats with ligated bile duct, glycuconjugates of Tx and TrIth are found in substantial amounts (10 to 13 per cent of total I), and it is probable that traces of these derivatives are normally present (143). One of them may be the unknown labelled substance present in greater amount in bile than in blood and which was detected before identification, of the glycuconjugates (144, 145, 146). In any case, in normal animals Harington's opinion, expressed 10 years ago, that circulation of peptides as hormone is "an unnecessary complication" (147), is confirmed by a series of new findings. Thyroglobulin appears in plasma only when thyroid function is disturbed (148); it has been found when very large amounts of I^{131} have been given, its abnormal secretion is then attributable to radiation effects on the gland (149). There remains, therefore very little of the concept of "protein iodine" of plasma formerly admitted in clinical chemistry, or of the so called D (diiodotyrosine) and T (thyroxine) fractions (150) supposedly carried by different protein fractions, since DIT and MIT were absent. Anyhow, as the circulating hormones precipitate quantitatively with protein reagents, the determination of the "protein iodine" is still of interest in the control of thyroid activity in pathological cases (151 to 156).

There is a striking difference between the composition of free iodinated

amino acids in thyroid extracts and in blood plasma. The proteolysis products of thyroglobulin contain nearly 60 per cent of total I in MIT and DIT and 25 to 30 per cent in Tx and TrITh. Between 75 to 90 percent of the plasma-organically bound I is included in the two latter which seem to be the only iodinated amino acids secreted by the gland. The choice made between the proteolysis products is a result of the dehalogenase activity only shown in plasma, specifically deiodination of MIT and DIT and not of Tx and TrITh. Thus, the mechanism of the secretion of thyroid hormones is governed by two enzymic actions: catheptases (157) which free all iodinated amino acids of thyroglobulin and dehalogenases (128) which eliminate the iodotyrosines, allowing the recovery of their iodine and the selective diffusion of Tx and TrITh. As a result of the endogenous utilization of iodine freed from MIT and DIT, it must be expected that nearly all iodine entering into the gland is secreted as Tx or TrITh, in part after one cycle, in part after a second or a large number of cycles.

Metabolism of thyroid hormones.—The metabolism of the thyroid hormones may be connected to their physiological effects if these are not attributable to a direct pharmacodynamic action, but to their degradation products. The latter hypothesis is not supported by facts but it remains attractive because of the delay necessary for the action of Tx. However, the derivative which is supposed to be formed during this delay is very probably not TrITh, because both hormones behave in the same way during the period necessary to exert their action (44, 158). Until now it has not been possible to propose even a crude scheme for the metabolism of Tx and TrITh, but their comparative behavior in the organism has been studied, chiefly with tracer doses of labelled I^{131} . A small part (10 to 20 per cent of injected iodine is found as iodide in urine within 24 to 48 hr. with a variable amount of unknown organic derivatives, possibly oxidation products of the alanine residue (159). Unaltered Tx or TrITh is found in feces (35, 142, 160) escaping to the enterohepatic circulation of hormones (161, 162). Very significant differences in the partition of I^{131} between urine and feces exist with different doses of hormones; the results obtained can be considered as physiological only with tracer doses (a few $\mu\text{g.}$ per rat). Larger amounts (10 to 100 $\mu\text{g.}$) are eliminated chiefly by a liver detoxication process, and pharmacodynamical doses (over 100 $\mu\text{g.}$) disturb its activity (163). Partition between organs and metabolic fate of Tx and TrITh must be considered.

Tracer doses of labelled hormones are not uniformly distributed in tissues. Liver accumulates I^{131} in a few hours after injection, more than any other organ. Gut and kidney also accumulate much less efficiently (164, 165). Posterior pituitary of rabbit collects traces, but anterior does not (166). Thus the special detoxication function of liver has been considered, and a number of papers have been devoted to iodinated constituents of bile. Glycuroconjugate of Tx is the most important of these (144, 145, 167, 168, 169), with free Tx. It is found 3 hr. after injection of Tx and is accompanied in large amounts by an unknown organic derivative and by traces of two others.

Iodides exist in small amounts. The deiodinating activity of liver is very small (rat, mice) on thyronine derivatives and even on DIT, or MIT and the liver function seems to be chiefly to conjugate and, eventually, to oxidize the alanine residue. Parallel qualitative results have been obtained with TrlTh (167). Comparison of bile excretion of both hormones shows differences: Tx is strongly conjugated, even when injected at 1 μ g. (rat). TrlTh is mainly eliminated free until doses of 10 μ g. are reached, possibly because of its lower blood level (163). The amounts of free and conjugated hormones after injection of 1 μ g. of the hormones can reach 70 per cent of the dose, that found in feces 20 to 30 per cent. Thus, the entero-hepatic circulation is physiologically very significant. Glycuroconjugates hydrolyze in the gut allowing resorption of a part of the hormone thus liberated. It may be that formation of conjugates, important even with tracer doses is physiologically scarcely active, being a delicate detoxication process. Long run experiments are necessary to establish this point.

The general metabolism of hormones in cells remains practically unknown. Labelled Tx injected can be recovered from tissue extracts with some unidentified organic iodinated compounds (160); the presence of TrlTh is doubtful. It is impossible to decide whether iodides are freed by partial deiodination of Tx or only from its degradation products. Iodine fixed at 3' in TrlTh or at 3',5' in Tx is more labile than at 3,5 (34, 46, 170), according to the general behavior of halogenated derivatives of thyronine (171). In the cases of TrlTh labelled on 3' and Tx on 3',5', the urine iodine loss, chiefly as I⁻ (160, 172, 173), proceeds at a higher rate and is more important with TrlTh than Tx. This is not necessarily bound to the effects of each.

Important work on these, chiefly on phosphate metabolism, is more in favor of a direct action of the hormone molecules. The difficulty to demonstrate *in vitro* effects of Tx has been one of the major obstacles in elucidating its mechanism of action. The actual tendency, supported by large number of results (174 to 178), is to explain it by interference in the phosphorylation process. An approach concerning its action by regulation of phosphokinase activity for the catalysis of phosphate transfer between ATP² and phosphocreatine (179) is probably concerned with a too limited metabolic field. The synergism of action on respiration by dinitrophenol and Tx on metabolism of the whole organism oriented research on the "control of the respiration, not at the level of the individual respiratory enzymes, but in the mitochondria, when respiration is coupled to the production of high-energy phosphate bonds" (180). The uncoupling effects of large amounts of Tx (4 mg. for 1 day and 8 mg. per day the 4 following days) to thyroidectomized rats on P/O ratio of liver slices or of mitochondria prepared from these is a very definite depression (177), i.e., a depression of phosphorylation of ATP by P² and an increase of oxidation rate. This has been found using various oxidation substrates, β -hydroxybutyrate, α -ketoglutarate, and glutamate (177), as with alanine incorporation into proteins of liver slices. Extraction of iodinated material after injection of labelled Tx shows that the cell nucleus

is poorer in it (181). Mitochondria and cell-fluid are richer and butanol-solubility corresponds to that of Tx. The only difficulty in making a conclusion on the physiological mechanism of hormone action is the severe thyrotoxicosis needed to observe these effects. It is not postulated that these findings are a result of a derivative of the hormone, and it is more likely they are attributable to the direct action of Tx on mitochondria enzymic systems coupling with respiration and synthesis of high-energy phosphate bonds (181). *In vitro* aerobic phosphorylation of rat liver mitochondria is influenced by Tx (10^{-4} to 10^{-5} M) as well as by dinitrophenols, leading to a diminution of P/O quotient. This effect is 3 times greater with L- than with D-Tx. The same observations have been made on mitochondria of Golden Syrian hamsters under the action of Tx or TrIth; complete inhibition of phosphorylation is frequently obtained for 10^{-4} M. A short reincubation with the hormone appears necessary, and fluoride reduces the effect.

Intensity factors affecting thyroxinogenesis.—We will only refer here to purely biochemical research, not to pharmacological or therapeutical, on the factors influencing the activity of thyroxinogenesis. It seemed from initial observations (182) that the thyrotropic hormone (TSH) of the anterior pituitary is able to act on the speed of the condensation reaction of DIT into Tx more than on the formation of DIT. This is not the case as, shown by more elaborate techniques applied to experiments with dogs (57), rats (183), and fishes (184). Hypophysectomy shortens all the steps of hormonogenesis. The contrary is observed by injection of TSH. Its influence is especially striking on important enzyme activities: proteolysis of thyroglobulin (157, 185, 186) and deiodination of MIT and DIT (187). The role of concentration of Tx and TrIth in posterior hypophysis of rabbit is unexplained (166, 188).

The concentration process of iodide by the gland is ruled by the plasma-iodide level (97, 98). It seems to be inhibited by some antithyroid drugs, as KSCN, but most of these, studied in other reviews (189, 197), act on utilization of I by the organ. The chief newly studied substances of this type have been mentioned before (91 to 94, 109). The level of circulating hormones is largely controlled by TSH² and the relative increase of iodide percentage of total blood I observed in hibernants during winter sleep (191) and in fishes [teleosts (192), elasmobranchs (193)] seems to be a result of low pituitary activity. A special reference must be devoted to amphibiotic migratory fishes, as salmon and sea trout. The total iodemia of these fishes is the highest yet observed on the high sea (up to 492 μ g. per 100 ml.) and is strongly reduced (10 to 20 μ g.) after a long stay in rivers (194). A series of important derivatives antagonizing Tx have been introduced into thyroid biochemistry under the name of antithyroxine substances (190, 195 to 201). They appear to be competitive inhibitors of the hormone owing to similarity of structure. Some are peripheral and direct inhibitors; others reduce TSH secretion, physiologically stimulated by Tx. This concept is too theoretical; antagonism is shown by many iodinated derivatives having no structural relation with thyronine. At the present time, the most studied substance of this series is the

N-butyl-ester of 4-hydroxy-3,5-diiodobenzoate (DIBB) (202, 203, 204). A daily dose of 25 mg. DIBB given to thyroidectomized rats inhibits all effects of a daily dose of 10 μ g. Tx, but has no action on doses of TrIth (205). This striking effect has been interpreted as indicating that DIBB inhibits specifically a hypothetical deiodination of Tx in TrIth and therefore, as an argument to consider the latter as the only substance acting on cells, as the real active hormone appearing in normal metabolism of Tx (173). The metabolism of this hormone is greatly stimulated by DIBB, mostly in its 3' and 5' substituents. Thus the action of the latter can be attributed to a destruction of Tx and not to a specific inhibition of a deiodinating process (172); it may be an activator of the degradation of Tx, not of TrIth in the condition of the experiment (tracer doses of Tx injected to thyroidectomized rats).

CONCLUDING REMARKS

In thyroglobulin, the formation and nature of iodinated amino acids, including the hormones, and the evolution of their biosynthesis is now reasonably well known. All other important problems opened up by the study of iodine metabolism in animals are in part unsolved. The chemical reaction leading to thyronine derivatives from iodotyrosines is only empirically known. The main processes in the metabolism of the two hormones and the mechanism of their action remain hypothetical. Thus even after the progress of the last decade, the biochemistry of the thyroid gland and of iodine remains promising, and still requires further research.

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METABOLITE ANTAGONISTS¹

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Since the subject of antimetabolites was last reviewed for this series by Winzler in 1949 (1), three books on the general subject (2, 3, 4) have been published. These, together with numerous reviews (5 to 10), provide a wealth of background material so that no general introduction to the subject is required here. In the present account, the literature from 1949 to November, 1953 has been surveyed with particular emphasis on the past two years. No attempt has been made to cover the literature completely. Rather, selected aspects of the subject have been emphasized and examined critically.

METABOLIC STUDIES

One of the most useful applications of metabolite antagonists has been found in the study of intermediary metabolism. In particular, antimetabolites which act as competitive antagonists of a metabolite by virtue of a close structural relationship to it are frequently highly specific. Consequently, the introduction of such antagonists into living cells can be expected to induce relatively specific responses, in contrast to the nonspecific effects of general protoplasmic poisons. With microorganisms, the combined use of various deficient mutants and antimetabolites has led to numerous fruitful studies such as those of Davis and his collaborators (11, 12). In higher animals, as Woolley (4) and others have demonstrated, it is frequently possible to induce or intensify specific deficiency symptoms. Any attempt at a logical organization of these metabolic studies is complicated by the interrelationships among the various metabolites which these same studies have helped to uncover. The relationship of PAB¹ to amino acids, purines, pyrimidines, folic acid, folinic acid (citrovorum factor, leucovorin), and vitamin B₁₂ illustrates the difficulty encountered in trying to classify antagonists. Some of the recent reports of new antagonists which do not involve metabolic studies are also mentioned briefly in this section.

Amino acids.—A relatively large number of amino acid antagonists have been examined [see Dittmer (13) for earlier references] and employed in the study of metabolic processes. Investigating the interrelationships involving aspartic acid with *Lactobacillus arabinosus* and *Lactobacillus casei*, Ravel *et al.* (14) found that the competitive inhibition of the utilization of this metabolite by cysteic acid was partially spared by threonine. Similar results with the former organism were also obtained with lysine. Unfortunately, no

¹ The following abbreviations are used in this chapter: ATP for adenosinetriphosphate; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; PAB for *p*-aminobenzoic acid; POB for *p*-hydroxybenzoic acid; RNA for ribonucleic acid.

definite conclusions could be drawn from these data, illustrating the weakness of the antimetabolite approach when used exclusively, and the desirability of combining it with other methods in order to obtain conclusive results. Roberts & Hunter (15) showed that both DL- β -aspartylhydrazide and DL- α -methylaspartic acid are effective inhibitors of the utilization of L-aspartic acid by *Leuconostoc mesenteroides*. The corresponding analogues of glutamic acid were much less active in this respect.

Borek & Waelsch (16) observed that β -hydroxyglutamic acid, as well as methionine sulfoxide and related sulfones and sulfoxides, blocked the utilization of glutamic acid for the synthesis of glutamine by *L. arabinosus*. Their studies indicated that glutamine is essential in the metabolism of this organism. Similar results with DL- β -hydroxyglutamic acid and DL- α -methylglutamic acid were obtained by Ayengar & Roberts (17), who found the activity to be limited to one of the diastereoisomers of the former antagonist.

Administration of ethionine to rats was shown by Lee & Williams (18) to inhibit the adaptive formation of tryptophan peroxidase normally stimulated by the administration of tryptophan. In adrenalectomized animals, L-methionine effected a partial reversal of the inhibition. The authors concluded that the antagonist interfered with the utilization of methionine for enzyme synthesis in the rat liver. Swendseid *et al.* (19) observed that ethionine caused a decrease in the activity of two enzymes, choline oxidase and sarcosine oxidase, when fed to rats. An enzymatically produced derivative of ethionine was suggested as the actual antagonist, although ethionine did inhibit the enzyme in rat liver homogenates.

Ryan (20) found δ -chloroleucine to be a good antagonist of L-leucine when studied with the spores of a leucineless mutant of *Neurospora crassa*. The inhibition index in this system was about 1:2, the antimetabolite interfering with the utilization of L-leucine. A homologue of lysine, ϵ -C-methyllysine (2,6-diaminoheptanoic acid) was reported by McLaren & Knight (21, 22) to inhibit the growth of *Streptococcus faecalis* and *L. mesenteroides*. L-Lysine reversed the growth inhibition of the racemic antimetabolite in a ratio of about 1:3.

The relationship between phenylalanine, tyrosine, and tryptophan has been the subject of numerous antimetabolite studies. Burckhalter & Stephens (23) found that the bacteriostatic action of DL- β -(*p*-aminophenyl)alanine could be reversed by either tyrosine or phenylalanine. Similarly, Lansford & Shive (24) reported that DL- β -(2-pyridyl)alanine and the corresponding 4-thiazolyl and 4-pyrazolyl derivatives inhibit the growth of *Escherichia coli*. This antibacterial action could be overcome by phenylalanine and also by tryptophan. The conclusion was drawn that the latter stimulates the synthesis of increased concentrations of the former in the organism. On the other hand, Atkinson *et al.* (25) were unable to demonstrate any reversal by tyrosine of the growth inhibition of *L. arabinosus* by DL- β -(*p*-fluorophenyl)alanine, although phenylalanine produced a competitive reversal. More ex-

tensive studies of this subject were carried out by Bergmann *et al.* (26) employing mutants of *E. coli* and antagonists which were considered to be more or less specific for one or another of the aromatic amino acids. On the basis of their investigations, these authors favored the following scheme for *E. coli* metabolism:

Precursor→tryptophan→phenylalanine→tyrosine→metabolic product of tyrosine.

This conclusion, which differs from the relationships proposed by Davis (12, 27), was based on sparing effects together with an interpretation of the action of several antimetabolites. For example, *p*-fluorophenylalanine was considered to be a specific inhibitor of the utilization of phenylalanine for conversion to tyrosine; *p*-amino-, *p*-nitro- and *m*-nitrophenylalanines were regarded as antagonists of the utilization of tyrosine, and *m*-nitrophenylalanine was also thought to interfere with the synthesis of tryptophan. While this type of investigation will be considered further (see Interpretation of Results), it does not seem to this reviewer than even the combined use of antimetabolites and mutant strains has, in this instance, succeeded in unraveling the complex interrelationships among the aromatic amino acids in a clean-cut manner. Incorrect assignment of antagonisms would, of course, lead to faulty interpretations.

Halvorson & Spiegelman (28) investigated the effects of a large number of different amino acid antagonists on adaptive enzyme formation in resting cells of *Saccharomyces cerevisiae*. A parallelism was found to exist between the capacity of an antimetabolite to inhibit growth and adaptation to maltose utilization by the yeast cells. When enzyme formation was suppressed by any one of the analogues, the incorporation from the amino acid pool not only of the corresponding amino acid, but of other amino acids as well, was inhibited. *p*-Fluorophenylalanine was the most potent of the inhibitors examined. The authors suggested that the absence of small peptides indicated the formation of complex peptides in the initial stages of enzyme synthesis. However, small peptides, if formed, might also be rapidly broken down again. In this connection, it is interesting to note that Dunn & Dittmer (29) found that when peptides were prepared by combining analogues and natural amino acids the latter could, in most instances, be utilized for growth by yeast cells.

p-Aminobenzoic acid (PAB), folic acid, and related metabolites.—In many respects the numerous interrelationships involving PAB are better known than for most other metabolites, primarily because of the availability of many specific antagonists. However, several very puzzling phenomena still remain unexplained. One of these is, of course, the failure of folic acid to reverse, noncompetitively, the antibacterial action of sulfanilamide derivatives except in isolated cases. The controversy over the mode of action of the sulfa drugs in relation to PAB and folic acid has continued with Sevag and his collaborators (30, 31, 32) ranged more or less against the field. Woods

(33) has reviewed the earlier evidence relating to this problem and more recent investigations in general confirm his original hypothesis. Folic acid antagonists have been reviewed by Jukes *et al.* (34) and by Petering (35).

The substance formed by sulfonamide-resistant strains of *Staphylococcus aureus* was identified as PAB by its isolation from the medium by Moss & Lemberg (36). Their observations were confirmed by Leskowitz *et al.* (37). A sulfonamide-resistant mutant of *E. coli* was found by Yaniv & Davis (38) to produce an enzyme with decreased affinity for PAB. Loss of resistance was frequently accompanied by a return to normal PAB requirements, although the two factors were not absolutely linked. A sulfonamide-resistant strain of *Streptococcus mitis* was discovered to require preformed folic acid for growth (39), whereas the parent strain did not. Lascelles & Woods (40) found that strains of *E. coli* and *Staph. aureus* synthesize a factor which can replace folic acid for the growth of *L. casei*. This factor was related to but not identical with folic or folinic acid. Sulfathiazole inhibited the synthesis, but was reversed competitively by PAB. A sulfonamide-resistant *Staph. aureus* synthesized much more of the factor than did the parent strain. An extensive study of the growth-inhibitory effect of sulfathiazole on 80 strains of bacteria was carried out by Möller *et al.* (41). These investigators found that the organisms could be divided into several classes depending on their growth requirements and the ability of folic acid to reverse sulfathiazole noncompetitively. In general, a folic acid antagonist, aminopterin (2-aminopteroylglutamic acid), inhibited the growth of only those organisms for which folic acid was effective in reversing sulfathiazole.

Sevag *et al.* (31) were unable to confirm the earlier report (42) of the growth-promoting or sulfonamide-reversing action of folic acid for *L. arabinosus* 17-5. These authors were of the opinion that the effects ascribed to this metabolite should be attributed to decomposition products such as PAB. However, their general conclusion, "the postulate that sulfonamides interfere with the synthesis of folic acid via *p*-aminobenzoic acid is not supported by the experimental facts reported here," is not justified. Sarett (43) concluded from his studies of the same system that sulfanilamide competitively inhibited the utilization of PAB for growth and decreased the amount of folic acid-like compounds formed. From the investigations of Hendlin *et al.* (44) it may be concluded that *L. arabinosus* constitutes an exceptional case. In their studies with *L. casei*, *S. faecalis*, and *L. citrovorum*, as well as *L. arabinosus*, these authors found that aminopterin blocked the conversion of folic to folinic acid in all instances. With sulfanilamide a partial block of this conversion was observed in *L. arabinosus*, while with aminopterin the inhibition was complete. Using PAB, on the other hand, it was found that sulfanilamide also completely prevented the formation of folinic acid. These results were considered to be in accord with the biosynthetic pathway, PAB→folic acid→folinic acid.

On the basis of their studies in rats, Nichol & Welch (45) suggested that aminopterin not only blocks the conversion of folic to folinic acid, but also

competitively inhibits the further utilization of the latter. A similar interpretation of their results was given by Goldin *et al.* (46), although they concluded that the inhibition may slowly become an irreversible one. In microorganisms and mice, Broquist *et al.* (47) found that folinic acid reverses the toxicity of aminopterin in a competitive manner. Weygand *et al.* (48) investigated the reduction products and formylated derivatives of aminopterin. They found the reduced formylated derivative to be a more active antimetabolite for *S. faecalis*, but for *L. citrovorum* aminopterin itself was more active. With *Drosophila* Hinton (49) found aminopterin to be a competitive antagonist of folic acid.

Antagonists of folic acid have proved valuable in elucidating the effect of this metabolite and folinic acid on the incorporation of one carbon fragments in the purines and other metabolites (50). Woolley & Pringle (51) found that aminopterin caused the accumulation of the same purine precursor, 4-amino-5-carboxamidoimidazole, which is also formed by *E. coli* when the organisms are exposed to PAB antagonists such as sulfadiazine. Sevag & Stewart (32) reported that while PAB reversed the growth inhibition of *E. coli* produced by sulfathiazole, it failed to prevent the accumulation of the imidazolecarboxamide in both control media and those containing sulfathiazole. Since the precursor accumulated also in the controls, the authors' conclusion that independent processes are involved appears to be unwarranted and is not in accord with numerous other investigations. Under similar conditions, Gots (52) found that PAB immediately relieved both the inhibition of growth and the accumulation of the carboxamide caused by sulfadiazine. However, the quantity already accumulated remained constant. Folic acid had no effect on either, but purines (adenine, guanine, xanthine) prevented accumulation almost completely without relieving growth inhibition. It was suggested that 4-amino-5-carboxamidoimidazole may be an abnormal by-product which is formed in the presence of the sulfonamide antimetabolites.

Using C^{14} -formate and bicarbonate, Skipper and co-workers (53) demonstrated that the incorporation of the former only into the nucleic acids of mice could be inhibited by aminopterin and 4-amino- N^{10} -methylpteroylglutamic acid (A-methopterin). The effect of A-methopterin was shown to be partially reversible by folic acid (54). Similarly, in the rat, Goldthwait & Bendich (55) found that the incorporation of C^{14} -formate into the purines and thymine was depressed by aminopterin to a greater extent than was N^{16} -adenine incorporation into the nucleic acids.

The studies of Broquist (56) also implicate folic and folinic acid in the synthesis of histidine by yeast cells. He found that folinic acid reversed competitively the growth inhibition of the yeast *Torula cremoris* produced by aminopterin. The toxicity of this antagonist could also be reversed non-competitively by the stepwise addition of methionine, methionine+purine bases and methionine+purine bases+histidine. With each addition the toxicity of aminopterin was successively diminished. Other approaches such

as tracer studies will be required to establish this relationship on a firm foundation.

A group of other substances too large to enumerate has been reported to be folic or folinic acid antagonists. Very few of these have been employed in metabolic studies. Hitchings and co-workers (57) investigated numerous 2,4-diaminopyrimidines which appear to be competitive antagonists for folic or folinic acid in many biological systems. The 5-phenyl-2,4-diaminopyrimidines proved to be competitive antagonists of folic acid for *L. casei*, and of folinic acid for *L. citrovorum*. With *S. faecalis*, a competitive inhibition of growth was observed over only a very narrow range of concentration of folinic acid. In general, the results with these antagonists were very similar to those obtained with aminopterin and suggested that the metabolite with which they compete may be more complex than either folic or folinic acid (58). A series of 1,2-dihydro-4,6-diamino-*s*-triazines was also found to possess antifolonic acid activity by Modest *et al.* (59). Reversal studies gave a complex picture, but with *S. faecalis* Foley (60) concluded that these compounds interfere with the conversion of folic to folinic acid. Resistant strains of this organism did not exhibit cross-resistance to aminopterin or the diaminopyrimidines (61).

Because of the varied responses obtained in different biological systems, it is evident from the preceding discussion that no clear-cut decision regarding the exact metabolic relationship of the various folic acid antagonists to folic and folinic acid can be made on the basis of present evidence. Both the diaminotriazines (59, 62) and pyrimidines (63) have shown considerable antimalarial activity, and these and other folic antagonists such as aminopterin have also been studied in the treatment of experimental tumors (35).

Pyrimidines, purines, nucleosides, and nucleotides.—The relationship of the purines and thymine or thymidine to folic and folinic acid has been referred to in the preceding paragraphs. An account of the earlier studies with various purine and pyrimidine antagonists has been given by Hitchings *et al.* (64). Again, the varied response in different biological systems, attributable in part to differences in the degree of interconvertibility, particularly among the purines, makes the metabolic studies with antimetabolites in this field unusually difficult to interpret (see also Metabolism of Antagonists). Many of the investigations of the pyrimidine and purine antagonists have been concerned primarily with their effects on neoplastic tissues (65, 66).

Snell & Cravens (67) found that thymidine partially counteracted the toxicity of aminopterin for the chick embryo. A combination with hypoxanthine desoxyriboside was more effective than thymidine alone, but in the absence of the latter, the former was ineffective. Thymine, hypoxanthine, folic acid, vitamin B₁₂ and concentrates of folinic acid were ineffective in counteracting the toxicity of aminopterin. With *L. leichmannii*, Weygand & Wacker (68, 69) showed that growth inhibition produced by 4-amino-5-methyl-2-thiouracil could be reversed in a noncompetitive manner by vitamin B₁₂. Using 5-bromouracil in this system, the antibacterial action was

reversed competitively by thymine or thymidine, but neither folic acid nor vitamin B₁₂ overcame its growth inhibitory action (69). However, Hitchings *et al.* (70, 71) found that both thymine and thymidine reversed competitively the growth inhibition of *L. casei* caused by 5-bromouracil, while the antagonist was without effect in a medium containing folic acid. Numerous complications were encountered in attempting to establish the locus of action of 5-nitrouracil and other pyrimidine analogues. A series of pyrimidine nucleoside antagonists was investigated by Visser and co-workers (72, 73, 74), who found that 5-chloro- or 5-bromouridine competitively inhibited the growth of a *Neurospora* mutant which required uracil, cytidine, or uridine. Based on studies with these and other related antimetabolites, it was suggested that uridine and cytidine may not be normal metabolic intermediates for nucleic acid synthesis in wild type *Neurospora*. Evidence was also obtained that the two nucleosides are not interconvertible. 5-Hydroxyuridine proved to be a competitive inhibitor of adenine and hypoxanthine for purine-requiring mutants of *E. coli* (74). Uridine or cytidine reversed the growth inhibition in a noncompetitive manner, although they did not satisfy the growth requirements of the mutants.

One hundred purine analogues were examined for their effects on *L. casei* by Elion and co-workers (75). Many of them proved to be antimetabolites, although ambiguous results were frequently obtained. The authors concluded that purine antagonists may be influenced in a variety of ways by the simultaneous presence of folic acid. In particular, it was found that the replacement of the hydroxyl group of guanine or hypoxanthine by a mercapto group led to the formation of potent antagonists which were reversible by purines as well as by folic acid. Balis *et al.* (76) studied the metabolism of 2,6-diaminopurine, an adenine antagonist for *L. casei*. Under the proper conditions the antimetabolite could also serve as a precursor of both polynucleotide adenine and guanine. The authors suggested that a primary requirement of the organism for adenine, possibly in the synthesis of DNA¹ or coenzymes, might explain the dual role of the diaminopurine. Their observations were not in accord with a primary inhibitory effect on polynucleotide synthesis or purine interconversion. The metabolism of a diaminopurine-resistant strain of *L. casei* was investigated by Elion *et al.* (77, 78). They found that in comparison to the wild strain, the mutant had a decreased ability to incorporate both adenine and the diaminopurine into pentose nucleic acids. The mutant strain was also resistant to other adenine antagonists such as purine and 8-azaadenine. In contrast to the results with the wild strain, the effects of a number of other antagonists were not blocked by adenine in the mutant. Elion *et al.* (79) also examined the purine metabolism of a 6-mercaptapurine-resistant *L. casei* strain. This organism had lost the ability to grow on hypoxanthine and grew only poorly on adenine. The results suggested that a metabolite containing hypoxanthine may be an intermediate in the conversion of adenine to guanine for *L. casei*.

Antagonists of other metabolites.—Metabolic studies with antagonists for

numerous other metabolites have been described in the past few years. In one of the very few instances where the mode of action of an antibiotic has been elucidated in convincing fashion, the substance has been shown to be a competitive antagonist for biotin. Umezawa *et al.* (80) found that the antibacterial activity of 4-thiazolidone-2-caproic acid for mycobacteria was specifically reversed by biotin with an inhibition index of about 2000. Synthetic biotin antagonists had been shown by Pope (81) to inhibit the growth of tubercle bacilli. Either synthesis or utilization was affected, depending on the antagonist.

While Gaebler & Beher (82) were unable to demonstrate any toxic effects from pyridine-3-sulfonic acid in normal or niacin-depleted dogs, they did observe signs of black tongue, as well as disturbances not reversible by niacin when depleted animals were fed 3-acetylpyridine. In fact, acute poisoning with the antimetabolite proved to be a condition quite different from uncomplicated black tongue. When 3-acetylpyridine containing excess C^{13} in the carbonyl group was employed, large amounts of N-methylnicotinamide containing excess C^{13} were excreted, indicating that a considerable proportion of the antagonist was oxidized to nicotinic acid (83). Beher & Anthony (84) ascribed the toxicity of 3-acetylpyridine to the inability of niacin-depleted animals to detoxify it as rapidly, as well as to its antivitamin activity. Coté *et al.* (85) found that pyrazinoic acid and its amide are nicotinamide antagonists for *L. arabinosus* but not for rats or chicks.

The point at which salicylic acid interferes with pantoic and pantothenic acids was investigated with *E. coli* mutants by Maas (86). Previous conflicting reports were resolved in favor of an inhibition of the synthesis of pantoic acid by salicylates. Subsequently, Maas & Vogel (87) found α -oxoisovaleric acid and α -oxo- β , β -dimethyl- γ -hydroxybutyric acid to be intermediates in the synthesis of pantoic acid for *E. coli* and *Aerobacter aerogenes*. These substances also reversed the growth inhibition produced by salicylic acid. Wieland *et al.* (88) investigated the effects of S-methyl-, S-ethyl-, and S-phenylpantetheine on the growth of *Lactobacillus helveticus*. Slight growth stimulation was noted at low concentrations, while at higher concentrations growth was inhibited. The S-phenyl derivative, in particular, was a good competitive antagonist of either pantothenic acid or pantetheine. A depression of steroid and fatty acid synthesis in liver slices was observed by Klein & Lipmann (89) in the presence of pantoyltauryl-*p*-anisidide. Partial reversal of these effects could be obtained with pantetheine but not with pantothenic acid.

Rabinowitz & Snell (90, 91) examined the activity of several pyridoxine antagonists with various microorganisms. 4-Desoxypyridoxine was without effect on organisms not requiring the preformed vitamin. Several yeasts and molds requiring an external source of pyridoxine were susceptible to the growth-inhibitory activity of the antagonist, but when grown under conditions enabling them to synthesize their own supply of vitamin, the same organisms were resistant. Pyridoxine was more effective in overcoming the

inhibition than the corresponding amine or aldehyde (90). Of several antagonists investigated, ω -methylpyridoxine (ethylpyridoxine) was in general the most potent followed by 4-desoxy and 5-desoxypyridoxine. The amine and aldehyde derivatives of 5-desoxypyridoxine were usually less inhibitory to growth. By the study of combinations, the authors concluded that the 4- and 5-desoxy derivatives acted by independent mechanisms, while ω -methyl- and 4-desoxypyridoxine gave additive effects (91).

L-Lyxoflavin was found by Shorb (92) to have considerable riboflavin activity when assayed with *Lactobacillus lactis*. With *L. casei*, however, the effect depended on the medium. Under certain conditions a competitive inhibition between the analogue and riboflavin could be demonstrated. Woolley (93) considered that 1,2-dichloro-4,5-diaminobenzene was an antagonist of the synthesis of both riboflavin and vitamin B₁₂ for a number of microorganisms not requiring an exogenous source of these growth factors. The growth-inhibiting action of the diaminodichlorobenzene was reversed competitively by 1,2-dimethyl-4,5-diaminobenzene as well as by higher concentrations of *o*-phenylenediamine. Riboflavin and vitamin B₁₂ were ineffective in overcoming the inhibition, leaving the author's conclusion in doubt. Using *L. lactis* as the test organism, Hendlin & Soars (94) found that the dimethyldiamine produced a growth inhibition which could be reversed competitively with vitamin B₁₂, while the growth inhibition produced by 5,6-dimethylbenzimidazole could not be prevented by the vitamin. A number of compounds related to 1,2-dichloro-4,5-diaminobenzene were investigated by Woolley & Pringle (95). In some instances, the antibacterial activity could be prevented by 1,2-dimethyl-4,5-diaminobenzene.

The competitive reversal of the antibacterial action of chlortetracycline by riboflavin was reported by Foster & Pittillo (96) with *E. coli* and a riboflavinless strain of *Bacillus subtilis*. Use of 1,2-dichloro-4,5-diaminobenzene to inhibit vitamin synthesis in combination with chlortetracycline led to a marked decrease in the minimum effective concentration of either for the inhibition of growth of the mutant. Curiously enough, the activity of oxytetracycline was unaffected by riboflavin, although the antibiotic did appear to inhibit the synthesis of the vitamin.

Woolley & Shaw (97, 98) investigated the activity of a number of serotonin analogues for their ability to prevent the constriction of segments of carotid arteries produced by the metabolite. A competitive effect was observed with 2,3-dimethyl-5-aminoindole. Injection of this antimetabolite into dogs prevented the hypertensive effect of serotonin. Administered orally this compound had only a partial effect, but 2-methyl-3-ethyl-5-nitroindole was active orally for dogs if given prior to the serotonin.

ANTAGONISM BETWEEN METABOLITES

Instances of antagonism between two metabolites have been known for a number of years, particularly among the amino acids (7). Like experiments with antimetabolites, the study of such antagonisms provides useful clues for

unraveling the mechanisms of various biosynthetic reactions. Here, too, restraint must be employed in the interpretation of results which should be combined with tracer studies and other methods before final conclusions are drawn.

It has also been suggested (4, 12) that such antagonisms between metabolites may comprise one of the normal regulatory mechanisms of living cells. In the extreme case, these antagonisms result in growth inhibition, while with more nearly physiological concentrations, control of enzymic synthesis or related processes may presumably be involved. The recent observations of Monod & Cohen-Bazire (99) and Cohn *et al.* (100) lend weight to this suggestion. The latter investigators found that an excess of methionine inhibited almost completely the formation of the enzyme system responsible for the synthesis of methionine by *E. coli*. None of the other amino acids tested showed such a striking effect on the formation of "methionine synthase," and none, including methionine, had a similar effect on the formation of β -galactosidase.

Most of the antagonistic effects among metabolites have been observed as reversible growth inhibitions of microorganisms in minimal media. Hirsch & Cohen (101) found that growth of a leucine-requiring mutant of *E. coli* was competitively inhibited by L-isoleucine. L-Valine, DL-methionine, and DL-norleucine were also inhibitory, while the D-isomers of valine and isoleucine were not. The peptides L-leucylglycine or glycyl-L-leucine also reversed, noncompetitively, the growth inhibition caused by L-isoleucine. The authors considered that a transpeptidation process might explain the lack of a competitive reversal with the peptides. Rowley (102) examined a large number of strains of *E. coli* which exhibited various amino acid antagonisms. The growth of many of the strains was inhibited by norvaline and norleucine and the inhibition could be overcome by leucine or methionine.

A mutant strain of *Neurospora*, which was inhibited by L-threonine, was described by Doudney & Wagner (103, 104). This growth inhibition could be overcome by DL-methionine, DL-homocysteine, DL-homoserine, and sulfanilamide. The methionine and sulfanilamide reversal was noncompetitive, and the effect of the latter could in turn be prevented by PAB. A combination of choline and the thiazole portion of thiamine also produced a noncompetitive reversal, particularly when combined with adenine and DL-serine at higher levels of threonine. The authors suggested that threonine competes with homocysteine in the formation of an intermediate which participates in the biosynthesis of methionine and the thiazole portion of thiamine. The synthesis of adenine and serine might also be affected indirectly. Such suggestions must be regarded as tentative until confirmatory evidence can be obtained.

At high concentrations, Davis (105) found that PAB inhibited the growth of *E. coli*. This effect was reversed competitively by *p*-hydroxybenzoic acid (POB). To a limited extent precursors of POB such as shikimic acid also re-

versed the growth inhibition. The rickettsiostatic effect of PAB has been explained on the basis of its interference with POB¹ metabolism (106).

With a double mutant blocked both before and after 5-dehydroshikimic acid, Davis (27) was able to demonstrate that this metabolite competitively interferes with the utilization of its own product, shikimic acid. Increasing ratios of 5-dehydroshikimic acid to shikimic acid prevented successively the conversion of the latter to tryosine, phenylalanine, tryptophan, PAB, POB, and an unknown sixth factor. A characteristic inhibition ratio was observed for blocking each of these syntheses, ranging from 0.2 for tyrosine to 30 for POB. The serial order of preferential synthesis was the same as that deduced by other methods (12).

Miller & Harrison (107) found that uracil inhibited growth of a yeast *S. cerevisiae*. Arginine specifically and competitively reversed the growth inhibition while none of the other amino acids tested did so. Other strains of yeast were not inhibited by uracil. Utilization of desoxyribonucleic acid or desoxyribonucleotides by *Lactobacillus bifidus* was found by Skeggs *et al.* (108) to be inhibited by ribonucleic acid and adenylic acid. Competition between the metabolites for a nucleotide phosphatase was suggested as a possible explanation for the inhibitory effects.

ENZYME INHIBITORS

Presumably practically all metabolite antagonists act in living cells by the inhibition of specific enzyme systems. In many instances, however, the enzymes involved are unknown. A few cases have been described in which the action of antimetabolites has been traced to inhibition of a particular enzyme system. Under the proper circumstances, such investigations can provide convincing evidence for the mechanism of action of antagonists. Studies with isolated enzyme systems, where possible, are also likely to be of considerable value in determining the mechanisms of action of drugs. Moreover, numerous other enzyme inhibitors may also find use as antagonists in living cells. In general, kinetic studies of various enzyme inhibitors such as those of Huang & Niemann (109) with α -chymotrypsin provide a useful background for the investigation of metabolite antagonists and illustrate some of the complications which may not be recognized in more complex systems.

Schou *et al.* (110) found that the enzymic synthesis of glutamohydroxamic acid from glutamine and hydroxylamine by a cell-free extract from *Proteus vulgaris* was inhibited by various amino acids, particularly glycine and L-aspartic acid. The amino acids inhibited competitively the utilization of glutamine in the enzymic exchange reaction. Both this system and the glutamine-synthesizing enzyme of sheep brain were shown by Pace & McDermott (111) to be inhibited by L-methionine sulfoximine. The former enzyme was much more sensitive to the inhibitor, and the authors suggested that a competition with glutamine was probably involved. Only L-methio-

nine sulfoximine was an effective inhibitor, and no reversing action could be demonstrated with *L*-methionine.

The investigations of Lichtenstein *et al.* (112, 113) established that the formation of the hydroxamic acid from *L*-glutamic acid with an extract of acetone-dried sheep brain and ATPⁱ was inhibited by DL-N-(γ -glutamyl) ethanolamine but not by DL- α -methylglutamic acid. In fact, the latter compound served as a substrate for the enzyme with the apparent formation of DL- α -methylglutamine. Using a sheep brain transferase system with *L*-glutamine and hydroxylamine as substrates, however, DL- α -methylglutamic acid acted as an inhibitor, while DL-N-(γ -glutamyl)ethanolamine did not affect the enzyme. Dog kidney glutaminase was also inhibited by the α -methylglutamic acid (113). This same substance was found by Roberts (114) to inhibit competitively the glutamic acid decarboxylase of *E. coli*.

Frieden *et al.* (115) examined the action of *L*-amino acid oxidase from cottonmouth venom on a number of amino acid antagonists. With the exception of analogues with a tertiary α -carbon atom, these substances were oxidized at rates very comparable to the natural amino acids. Similar results were obtained with hog kidney D-amino acid oxidase, although the rates were frequently slower. The authors suggested that these amino acid antagonists did not act by inhibition of the enzymic degradation of amino acids.

A partially purified enzyme from brewer's yeast which catalyzed the phosphorylation of adenosine by ATP was found by Kornberg & Pricer (116) to act also on 2,6-diaminopurine riboside. The product of the reaction in the latter case was 2,6-diaminopurine riboside-5'-phosphate. When coupled with other enzyme systems, the triphosphate analogue of ATP was formed. Clarke *et al.* (117) also found that 2,6-diaminopurine riboside was the only compound of those examined, other than adenosine, which was deaminated by adenosine deaminase.

Zatman *et al.* (118) demonstrated the conversion of isonicotinylhydrazide to an analogue of DPNⁱ by an enzyme from pig brain. The crude product was shown to contain the hydrazide in place of nicotinamide by analysis and formation of free hydrazide on hydrolysis. This product was at least twice as active an inhibitor of beef spleen diphosphopyridine nucleotidase as isonicotinylhydrazide. The same enzyme from other sources was much less sensitive to these inhibitors. Williams (119) found that adenine, adenosine, and ATP were competitive inhibitors of DPN utilization by malic dehydrogenase. The author considered that the results might explain the symptoms of canine black tongue produced by feeding adenine.

The effect of 4-desoxypyridoxine on tyrosine decarboxylase from *Strep. faecalis* was investigated by Umbreit & Waddell (120). With a crude enzyme preparation, they found that the antimetabolite did not compete with excess pyridoxal for conversion to its phosphate in the presence of ATP. When pyridoxal was limiting, however, then 4-desoxypyridoxine was also converted to the phosphate. With an impure sample of 4-desoxypyridoxine phosphate added first or simultaneously with the coenzyme, inhibition of tyrosine de-

carboxylase resulted. The authors considered that these observations explained why the antagonist produced symptoms of a B₆ deficiency only in animals on a restricted intake of the vitamin. Studies with other enzyme systems will be required to confirm and extend this explanation.

Evidence for the phosphorylation of arabitylflavin and dichloroflavin by yeast flavokinase has been described by Kearney (121). The rate of phosphorylation of the latter was slightly greater than for riboflavin itself. Iso-riboflavin and several other analogues were not phosphorylated, while lumiflavin was the only one examined which inhibited the phosphorylation of riboflavin.

Ensebi & Cerecedo (122) observed that oxythiamine pyrophosphate inhibited pyruvate decarboxylation by a purified yeast enzyme. Oxythiamine monophosphate and pyrithiamine were inactive in this system. Similar results with yeast apocarboxylase were obtained by Velluz & Herbain (123) using oxythiamine triphosphate. Free oxythiamine did not produce inhibition under the same conditions. Onrust *et al.* (124) showed that animal pyruvic oxidase was also inhibited completely by oxythiamine triphosphate. Woolley (125) studied the action of a crude preparation of pyrithiamine pyrophosphate on the combination of thiamine pyrophosphate with yeast apocarboxylase. He found that these two pyrophosphates competed for the apoenzyme. An attempt to establish the mechanism of action of pyrithiamine (neopyrithiamine) itself using cell-free chicken blood led to inconclusive results.

The well-known toxic effects of various phosphate and pyrophosphate esters in animals, as a result of the inhibition of cholinesterase, has led to extensive investigations of the mechanism of action of these enzyme inhibitors. The studies of Wilson (126, 127), Augustinsson & Grahn (128), Aldridge (129), and others have indicated that the slowly reversible nature of the inhibition by esters such as tetraethyl pyrophosphate and dimethyl-*p*-nitrophenylphosphate is probably attributable to an actual phosphorylation of the enzyme by the inhibitor. Hydroxylamine reacts with the enzyme phosphate to yield reactivated enzyme and hydroxylamine phosphate (128). Considerable evidence concerning the nature of the "active centers" of the enzyme has also resulted from these and similar studies.

The reactions to tetramethylthiuram disulfide following the ingestion of ethanol have been shown to be a result of the inhibition of various enzyme systems which oxidize acetaldehyde. Thus, Graham (130) found that acetaldehyde dehydrogenase from rat liver was strongly inhibited by low concentrations of this compound which acted as a competitive inhibitor of DPN. Reduced glutathione reversed the inhibition in relatively low concentrations. Nygaard & Sumner (131) demonstrated that tetramethylthiuram disulfide also inhibited D-glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle when either acetaldehyde or D-glyceraldehyde was the substrate. A competition between inhibitor and substrate was observed. The apparent dissociation constant for the enzyme-inhibitor complex was $5 \times 10^{-6} M$.

Following earlier investigations, Roblin and co-workers (132, 133) studied

a group of heterocyclic unsubstituted sulfonamides as carbonic anhydrase inhibitors. Based on the assumption of a competitive inhibition, a correlation between the acid dissociation constants of these compounds and their effectiveness as enzyme inhibitors was observed. Aside from its therapeutic uses, one of these potent inhibitors, 2-acetyl-amino-1,3,4-thiadiazole-5-sulfonamide, has been widely used in elucidating the functions of carbonic anhydrase *in vivo*.

Fellows & Bernheim (134) observed a relationship between the stimulatory effect on motor activity in rats and inhibition of amine oxidase with tyramine as the substrate. With some exceptions, a number of aralkyl amines examined showed a direct correlation. Hydrazinophthalazine and related compounds were reported by Gross *et al.* (135) to inhibit diamine oxidase, although the authors did not observe any relationship between effects on blood pressure and enzyme inhibitory activity. Numerous other substances have been found to inhibit the amine oxidases. Schuler (136), Blaschko *et al.* (137), and Zeller and co-workers (138, 139) have described a number of these.

Based on earlier results, Hofstee (140) and Petering & Schmitt (141) examined the effect of a number of pterines on xanthine oxidase from milk or rat liver. A competitive inhibition between the substrate, xanthine or xanthopterin, and the pterine was demonstrated (140). In particular, 2-amino-4-hydroxy-6-hydroxymethylpterine was shown to be about as active as the 6-formyl derivative, complete inhibition of the enzyme being observed at an inhibitor/substrate ratio of 1/1000 with either (141). These same pterines were also found by Dietrich & Shapiro (142) to be effective inhibitors of crude mouse liver guanase. The inhibition was dependent on preincubation of enzyme with inhibitor before addition of substrate. Shapiro *et al.* (143) utilized this inhibitory effect in an effort to protect 8-azaguanine from the action of guanase which converts it to inactive 8-azaxanthine (144).

Shaw & Woolley (145) observed that 2-azaadenine and 2-azahypoxanthine served as substrates for milk xanthine oxidase. The product of the reaction of the former antagonist was isolated and found to be 8-hydroxy-2-azaadenine. 2-Azaadenine inhibited the oxidation of xanthine by competing with it for xanthine oxidase, but the antimetabolite was oxidized approximately five times as rapidly.

The classical inhibition of succinic dehydrogenase by malonic acid was reinvestigated by Pardee & Potter (146). They showed that in addition to the oxidation of succinate, malonate also inhibits the oxidation of oxalacetate by fortified homogenates. The latter inhibition was dependent on the concentration of Mg^{++} and could be explained by the formation of a complex of malonate with free and bound Mg^{++} . Busch & Potter (147) found that the injection of malonate into rats caused the accumulation of succinate in various tissues. A number of other inhibitors of succinic dehydrogenase have been described (148, 149). Although it has no effect on the enzyme in rat liver homogenates (148), Seaman (150) found that arsonacetate competitively inhibits the succinic dehydrogenase activity of *Tetrahymena geleii*. The

quinquevalent rather than the reduced arseno form was the active inhibitor, and the sulfhydryl groupings of the enzyme were not affected.

METABOLISM OF ANTAGONISTS

Instances in which antagonists are metabolized by living cells to less complex substances have been reported from time to time. In addition, cases in which antimetabolites such as α -methylglutamic acid, desoxypyridoxine, and dichloroflavin appear to serve as substrates for isolated enzymes, with their conversion to more complex compounds analogous to those formed from the normal substrates, are recorded under Enzyme Inhibitors. In recent years, it has become apparent that this same phenomenon may sometimes, although by no means always, occur also in living systems. Several examples of this type of metabolism of antagonists are described in the succeeding paragraphs.

Liébecq & Peters (151) suggested that the inhibition of oxygen uptake in various tissue homogenates by fluoroacetate might be attributable to the formation of a more complex antimetabolite. In rats given fluoroacetate, citrate accumulated, although no inhibition of the enzyme aconitase could be demonstrated *in vitro* (152). Further evidence was provided by Elliott & Kalnitsky (153) and Massey & Rogers (154), who found that preincubation of fluoroacetate with tissues gave much more complete inhibition of oxygen uptake. The former observers demonstrated a competitive inhibition of citrate oxidation and identified fluorine in the citrate fraction. Lotspiech *et al.* (155) isolated the crude product from tissues poisoned with fluoroacetate and demonstrated that it inhibited the various reactions of aconitase. Final proof was provided by Peters *et al.* (156, 157), who purified fluorocitric acid from the kidneys of animals receiving fluoroacetate and established its identity with synthetic fluorocitric acid.

The metabolism in rats of ethionine labeled with C^{14} in the methylene carbon of the ethyl group and with S^{35} was investigated by Stekol & Weiss (158). Their data suggested that ethionine sulfur was available for the synthesis of cysteine and that the ethyl group of ethionine participated in the synthesis of ethyl analogues of various metabolites. Growth inhibition of rats caused by ethionine was attributed to the latter (159). Levine & Tarver (160) also studied the metabolism of C^{14} -ethionine with respect to its incorporation into the proteins of rats. They concluded that such incorporation with the formation of abnormal proteins did occur since ethionine could not be washed out of the proteins, the C^{14} -ethyl group was split off with HI, and the uptake was much greater than could be accounted for by adsorption. As the authors pointed out, further work will be required to provide conclusive evidence.

Dried cells of *Strep. faecalis* which had been grown in 5-bromouracil- Br^{82} were found by Weygand *et al.* (161) to have retained appreciable quantities of antagonist which could not be eluted by washing with water. Thymine prevented both the growth inhibition and the uptake of the antagonist in a

competitive manner, while folic acid reversed the growth inhibition noncompetitively, but did not affect the uptake. Subsequently, it was shown (162) that the 5-bromouracil-Br⁸³ was incorporated in the nucleic acid fraction. With *Enterococcus stei*, Weygand & Wacker (163) found that, although growth was not inhibited, the antagonist was taken up in much the same manner.

Jeener & Rosseels (164) observed that the effectiveness of thiouracil as a possible competitive inhibitor of uracil in the multiplication of tobacco mosaic virus (165) was dependent on the size of the inoculum. The results suggested a greater inhibition if the antimetabolite acted earlier in the infection, and led to a study of the action of S³⁵-thiouracil in concentrations sufficient to reduce the multiplication of virus to 50 per cent. The ribonucleic acid, obtained from the virus after it had been recrystallized three times, contained activity equivalent to 20 per cent of the normal uracil content. Hydrolysis of pyrimidine nucleotides led to the formation of a component which differed from normal nucleotides, and it was suggested that this component was thionucleic acid. On complete hydrolysis, only 20 per cent of the S³⁵-thiouracil could be isolated. Resting cells of *S. faecalis* which hydrolyze thymidine were found by Prusoff (166) to convert azathymine to what appeared to be azathymidine. The rate of hydrolysis of thymidine was unchanged, but at the same time the antagonist was apparently converted to the desoxyriboside.

Heinrich *et al.* (167) isolated 8-azaguanine from the RNA¹ of *Tetrahymena geleii* grown in the presence of the antagonist in concentrations allowing half maximal growth in a complete medium. In a uracil deficient medium in which the antagonist showed no inhibition of growth, no incorporation could be detected. Mitchell *et al.* (168) had previously found evidence for the incorporation of 8-azaguanine-C¹⁴ into the nucleic acids of tumors and viscera of tumor-bearing mice treated with the compound. Similar results were obtained by Mandel *et al.* (169), who demonstrated that hydrolysis of the RNA fraction to the nucleotide stage produced no active antagonist whereas subsequent hydrolysis to the free purines did, thus establishing its presence in chemical combination. It was concluded that 8-azaguanine does not act as a simple antimetabolite of guanine in the synthesis of nucleoprotein. In an 8-azaguanine-requiring strain of mouse leukemia, Bennett *et al.* (170) found that the incorporation of the compound into DNA and RNA was only about 1/100 as great as in the normal strain. Matthews (171) showed that 8-azaguanine was also incorporated into the nucleic acids of tobacco mosaic virus. Paper electrophoresis provided evidence for the presence of 8-azaguanic acid after mild alkaline hydrolysis. The author suggested that the incorporation of the antimetabolite into the nucleic acids rendered the virus particles "sterile".

While in only one instance has complete chemical proof been established, these results and others not included here seem to provide reasonable evidence that in certain cases antagonists can participate in the normal metabolic

processes of the cell. In so doing, however, a point is eventually reached where growth inhibition ensues because the cell is unable to carry out its normal functions with the metabolite analogue formed from the original antimetabolite.

INTERPRETATION OF RESULTS

Some of the factors which complicate the interpretation of the results obtained with metabolite antagonists are well illustrated in the study of isolated enzyme systems (see Enzyme Inhibitors). Thus, an inhibitor competing with the normal substrate may undergo the same type of reaction, being converted to either an inactive product or a more complex inhibitor such as an analogue of a coenzyme. Alternatively, the inhibitor may undergo no reaction but still compete with the substrate or a coenzyme. The inhibitor may react chemically with the enzyme or the substrate. It may inhibit two or more different enzyme systems simultaneously. Moreover, an effective inhibitor of an enzyme from one source may have little or no effect on what otherwise appears to be the same enzyme derived from a different source. Studies of the kinetics of enzyme inhibitions have demonstrated the existence of still other complexities under conditions which are far simpler than those encountered in living cells (172).

Turning now to antagonists in living cells, the interpretation of results is further complicated by the many interrelationships of enzyme systems as well as other factors, such as the penetration of cell walls. For example, Cramer & Woodward (173) found that the anaerobic fermentation of glucose by yeast cells was inhibited by 2-desoxy-D-glucose in a competitive manner. On the other hand, the fermentation in cell-free extracts was not inhibited initially by the antimetabolite. The authors concluded that 2-desoxy-D-glucose competitively inhibited a cell-wall enzyme essential for the transport of glucose into the cell.

Moore & Boylen (174) distinguished at least three types of growth inhibition when the effects of various antagonists were examined in terms of growth rates. By plotting the number of organisms in the logarithmic phase of growth as a function of time, varying concentrations of several antimetabolites such as 5-bromouracil gave straight line relationships corresponding approximately to the steady state equation, $n_t = n_0 2^{kt}$, where n_t is the number of organisms at time t , and n_0 is the initial inoculum, k being the rate constant. Sulfanilamide, on the other hand, gave growth curves with two linear portions having different slopes. A lag phase, possibly corresponding to the time required for the degradation or dilution of a coenzyme or other growth factor, was observed. Under these circumstances, the simple theory was no longer adequate. In the case of pyridine-3-sulfonic acid a striking effect of inoculum size was found. With large inocula, after a delay, the rate of growth was essentially the same as the controls, while with smaller inocula the cultures eventually became sterile. Conclusions based on the degree of inhibition of growth at some arbitrary time might, in such a case, depend primarily on

the time selected. The authors emphasized the importance of investigating growth rates in detail, particularly when the interpretation of reversal studies is involved.

The kinetic studies of McRae, Foster & Bonner (175, 176, 177) with plant auxins demonstrate the value of quantitative results in the interpretation of the mechanism of action of antagonists. Using classical enzyme kinetics, it was found that typical plots of $1/V$ versus $1/S$ gave excellent agreement with a true competitive inhibition for antiauxins such as 2,6-dichloro-, and 2,4,6-trichlorophenoxyacetic acids (175). Furthermore, the inhibition of growth by high concentrations of auxins such as indoleacetic acid could be explained on the basis of a two point attachment to a specific receptor site in the plant (176). As the authors point out, multiple attachment, with the formation of bimolecular or higher inactive complexes, may also explain the inhibitory action of other metabolites at unphysiologically high concentrations.

The same substance can sometimes act as an antagonist of two or possibly more metabolites as illustrated by methionine sulfoximine, $\text{CH}_3\text{SO}(\text{NH})\text{CH}_2\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$. With *L. mesenteroides*, Heathcote & Pace (178) reported this compound to be a glutamine antagonist. The inhibition ratio was approximately 1:10 for this system, where the antagonist presumably interferes with the utilization of glutamine. Heathcote (179) had previously found the growth inhibitory action of the sulfoximine on the same organism to be reversed by methionine in a ratio of about 1:50.

Davis (105) observed that, particularly in the presence of DL-aspartic acid, *p*-nitrobenzoic acid was an antagonist of both PAB and POB for *E. coli*. Under certain conditions, neither metabolite alone reversed the growth inhibition produced by the antagonist. Subsequently, Davis & Maas (180) made use of this double antagonism to analyze the mechanism of resistance to *p*-nitrobenzoic acid in mutants of *E. coli*. They found that strains resistant to either of these inhibitions were not resistant to the other. Consequently, the decrease in sensitivity could not be attributable to decreased penetration or increased destruction of the antagonist. Little cross-resistance with other PAB or POB antagonists such as sulfathiazole or 4, 4'-dihydroxydiphenyl sulfone was observed. After ruling out a number of other possibilities, in this particular case it was concluded that the results were all compatible with the presence of altered enzymes with decreased relative affinity for the inhibitor compared with one or the other of the two metabolites. It is evident that such results could easily have been misinterpreted had a less complete study, unsupplemented by the use of resistant mutants, been employed.

Another complication bearing on the interpretation of results is illustrated in the studies of Kihara & Snell (181). These investigators found that *L. casei* grows in the absence of pyridoxine if D-alanine and certain peptides containing L-alanine are supplied. Further investigation established that D-alanine, which is itself essential for growth in the absence of pyridoxine, inhibited the utilization of L-alanine. While L-alanine itself was ineffective in

overcoming the growth inhibition, L-alanine peptides served this purpose, although they were hydrolyzed by resting cells to the free amino acid. It was pointed out that, while the L-alanine peptides in this instance appear to serve as precursors of L-alanine, they are more effective than the latter in overcoming the inhibitory action of the antagonist. This is, of course, contrary to the usual relationship between metabolite and precursor in overcoming the action of an antimetabolite.

Occasionally a chemical reaction between metabolite and antagonist may give the appearance of a competitive antagonism. Yoneda & Asano (182) investigated the inhibitory action of isonicotinylhydrazide on the decarboxylation of arginine by resting cells of *E. coli*. They found that pyridoxine or pyridoxal in the same molar concentrations as the inhibitor restored part of the activity of the cells, the latter being considerably more effective. Since the resting cells could presumably convert pyridoxine to the aldehyde, the possibility of a direct chemical reaction between pyridoxal and the hydrazide has not been excluded in this instance.

As has been pointed out previously (9), making more than one structural alteration at a time, in attempting to convert a metabolite to an antagonist, usually leads to less and less active antagonists with respect to the original metabolite. This conclusion is consistent with the results obtained with folic acid antagonists (34, 35) as well as the studies of Visser *et al.* (183) with anti-metabolites of uridine, in which two structural alterations were made. Woolley and co-workers (95, 184, 185) have considered that such multiple alterations lead to irreversible, although presumably specific, antagonisms. Such speculations do not appear to be particularly fruitful, since they cannot be established experimentally. Moreover, equally possible alternative explanations are available.

On the basis of a remote relationship involving four structural alterations, Woolley (186) suggested that chloramphenicol is a phenylalanine antagonist. When *E. coli* was the test organism, this antagonism was noncompetitive and could be demonstrated only over approximately a two-fold range of concentrations in a particular medium. While this slight effect was confirmed by Molho & Molho-Lacroix (187), these authors assumed that chloramphenicol is a peptide antagonist. In any event, the literature is replete with examples of a slight noncompetitive reversing action of growth inhibitors by various metabolites in minimal media, as well as by unknown factors in complex media. Even substances that have no known metabolic function have been shown to reverse the antibacterial action of chloramphenicol, as illustrated by the studies of Smith (188). Such compounds as *m*-nitrobenzaldehyde and 2-amino-4-nitrophenol were found to be highly active in reversing the effect of the antibiotic on *E. coli*.

Instances have also been recorded in which a close structural relationship did not lead to an antagonism between a metabolite and its analogue. For example, Erlenmeyer & Lehmann (189) found that, while 2-methyl-4-amino-hexanone-5, $\text{CH}_3\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}(\text{NH}_2)\text{COCH}_3$, a ketone analogue of leu-

cine, inhibited tail regeneration in *Xenopus* tadpoles over a relatively wide range of concentrations, leucine was ineffective as a reversing agent.

These results certainly suggest that, in the absence of a competitive antagonism which can be demonstrated over a wide range of concentrations, a conservative approach to the interpretation of results is essential. However, the absence of such a criterion does not always, in itself, eliminate the possibility of a specific antagonism. In certain biological systems, the growth inhibitory action of aminopterin is reversed only over a very small range of concentrations by folic acid (34). With other systems, this is not the case (49), and folinic acid is sometimes considerably more active as a reversing agent (190). While from a structural standpoint aminopterin is much more closely related to folic acid, if, in certain systems, it were converted to an analogue of folinic acid, then the overall result would indicate it to be an antagonist of the utilization of the latter metabolite. Although such a conversion does not appear to have been demonstrated in the case of folic acid, there are numerous analogies for such a possibility (see Metabolism of Antagonists). In general, the conversion of an antagonist to a more complex antimetabolite can obviously lead to difficulties in the interpretation of results.

An inherent weakness in the use of metabolite antagonists in metabolic studies has been emphasized previously (9, 27, 64), as well as in this review. Sparing effects, noncompetitive reversals and similar phenomena observed with various metabolite-antimetabolite combinations frequently provide valuable new clues in tracing pathways in intermediary metabolism. However, because of the complex nature of many antagonisms in living cells, an unequivocal interpretation based exclusively on these results can rarely be made. On the other hand, if followed by tracer studies and other methods of confirmation, the leads provided by antimetabolite studies are sometimes uniquely valuable.

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NONOXIDATIVE AND NONPROTEOLYTIC ENZYMES^{1,2}

BIOSYNTHESIS AND METABOLISM OF PHOSPHORUS COMPOUNDS

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This section deals mainly with a discussion of some important aspects of phosphorylations and dephosphorylations which have grown out of recent studies, and is restricted to basic phosphorylation phenomena. The coupling between oxidations and phosphorylation will be referred to briefly, since there are sections elsewhere dealing with oxidation reductions (7) and with carbohydrate metabolism (323).

FUNDAMENTAL ISSUES CLARIFIED BY ISOTOPE TECHNIQUES

Progress in our understanding of the mechanism of enzymic phosphorylations can be partly ascribed to the fundamental experiments by Cohn, using O¹⁸-enriched phosphate as well as O¹⁸-enriched water. Her studies (62) on the enzymic and nonenzymic splitting of α -glucose-1-phosphate illustrate very well some of the main features of phosphorolysis, phosphorylation, and dephosphorylation. The main outcome of these studies, it will be recalled, was that the polysaccharide phosphorylase catalyzes a split of the phosphate linkage between the carbon and the oxygen. This is a clear indication that this type of enzyme catalyzes the transfer of glycosyl residues and not phosphoryl residues. This agrees with the fact that sucrose phosphorylase is also a transglycosidase and that nucleoside phosphorylase can make a ribosyl phosphate from a ribosyl C—N linkage. Likewise, acid hydrolysis

¹ The survey of the literature pertaining to this review was completed in December, 1953.

² The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for Coenzyme A; DNP for 2,4-dinitrophenol; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); FAD for flavine-adenine-dinucleotide; GDP for glucose diphosphate; GSH for glutathione (reduced form); IDP for inosinetriphosphate; IMP for inosinemonophosphate; INH for isonicotinic acid hydrazide; ITP for inosinetriphosphate; MDP for D-mannose-1,6-diphosphate; RNA for ribose-nucleic acid; TCA for trichloroacetic acid; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); UDP for uridinediphosphate; UDPG for uridinediphosphoglucose; UTP for uridinetriphosphate.

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brings about a splitting of the C—O linkage. Lately it has been possible to observe formation of oligosaccharides during acid hydrolysis of disaccharides.

In the splitting of glucose-1-phosphate by an alkaline phosphatase the O—P and not the O—C linkage is "under attack" [Cohn (62); Clarke & Koshland (59)]. In other words, a "phosphoryl" is formed which subsequently takes up water. This mode of action of phosphatase shows why phosphatases are also able to catalyze transphosphorylations ([Morton (232)]. In phosphorylations, especially in the so-called ATP² kinases in which the terminal phosphoryl is transferred to a hydroxyl acceptor (ROH) such as glucose or choline, there is again the possibility of a direct cleavage of a O—P linkage by ROH in which no exchange with water would occur or a formation of a complex di-ester with ROH succeeded by a cleavage with water. If the water was enriched with O¹⁸ in the latter case, either the ROP ester or the nucleotide (ADP²) should have taken up O¹⁸ (See section "Hydroxyl Kinases").

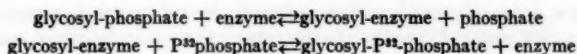
Prospects in connection with techniques, using O¹⁸-enriched phosphate (or phosphoric esters) or water, include the following: (a) Identification of a transitory phosphorolysis of linkages (brought about by O¹⁸-enriched phosphate). This type of reaction, even after a loss of the phosphate group (provided it is attributable to an O—P fission as in a phosphatase action) would have left a permanent mark, an O¹⁸ atom in the organic structure. Examples: (i) phosphorolysis of glycosyl linkages, (ii) phosphorolysis of acyl-mercapto linkages, (iii) phosphorolysis of CoA²—S—Enzyme. In this way more or less complex polysaccharides or definite groups in proteins can be exposed as "business centers" even after the phosphate has been lost by phosphatase action. Replacement reaction with other groups can of course be similarly detected.

(b) Exposure of hitherto indistinguishable types of pathways in phosphorylations. By the use of O¹⁸-enriched water it is feasible to detect transitory di-ester formations in transphosphorylations. A direct transphosphorylation would not give rise to an O¹⁸-enrichment of any of the reaction products.

The application of O¹⁸ is in many ways the ideal technique for the solution and clarification of many fundamental problems in enzymology, but the technique is complex and time-consuming.

This year's publications dealing with phosphorylations impress the reader with the popularity of the concept of enzymes as acceptors of anhydride groups. It has been postulated for instance that enzymes can be phosphorylated, "adenylated," exchange with glycosyl compounds, etc. As emphasized by Zatman *et al.* (332) this idea dates back at least 20 years to Langenbeck. It was revived through the studies by Doudoroff, Barker & Hassid on sucrose phosphorylase. Although the idea may be immensely fertile, it must be born in mind that at the present time the assumption that groups in the enzyme protein form covalent linkages with groups from the substrate, however attractive the hypothesis may be, rests so far solely on indirect observations. These indirect observations are in themselves of interest because many closely related enzyme reactions behave completely differently. Thus,

sucrose phosphorylase catalyzes a rapid exchange between free phosphate and α -glucose-1-phosphate, and the addition of the other glycosyl acceptor, fructose, does not speed up the exchange; on the contrary it exerts a slight inhibition. How different is the situation in polysaccharide or maltose phosphorylase; here no exchange between phosphate and 1-ester phosphate takes place until the other glycosyl acceptor is added. In a long series of hydrolytic enzymes it has been observed that besides the irreversible loss as a result of hydrolysis, there is an exchange between one of the hydrolysis products, as a general rule that product which does not pick up water—OH, and the same group in the original reactant. This type of reaction has also been interpreted as passing through a transitory anhydride-enzyme covalent compound the linkage of which is broken by the original nondehydrated partner. As an example:



In general the occurrence of such an exchange reaction, whether it be caused by an enzyme of hydrolytic activity or by one with a purely transfer activity, is the first prerequisite for the existence of a "product-anhydride-enzyme compound" but may not necessarily be the only possibility.

Whatever the mechanism may be for the exchange reaction in hydrolytically active enzymes, we must also try to explain how a reaction which under the conditions of the experiment releases only a small amount of free energy, or even consumes free energy, can run "successfully" side by side with a hydrolysis which is accompanied by a release of large amounts of free energy. Here the only possible approach seems to be kinetic. The reviewer (cf. 147) has considered the possibility that the activation energy of the transfer reaction is lowered to a much greater degree than that of the hydrolysis. If a sufficient time is permitted, or if the temperature is raised adequately, the final balance will presumably be entirely in favor of hydrolysis.

The role of metals in enzymic phosphorylations is still obscure. Divalent cations, calcium, magnesium, and manganese play the predominant role in these reactions. A new technique [the microwave paramagnetic resonance absorption method (Cohn (62))] has made it possible to state with certainty that phosphoric esters, including nucleotides, form covalent complexes with manganese, one of the most universally active ions in phosphorylations.

The concepts of the term as well as the concept "phosphate bond energy" has been under attack by Gillespie *et al.* (99). Aside from a justified request for unambiguous terminology and more emphasis on factors such as concentration effects ([cf. also Linderstrøm-Lang (201a); Lipmann (204)], some of the other objections raised by Gillespie seem less justified. Lipmann's emphatic differentiation between "energy-rich" and "energy-poor" phosphoric esters was purposely accentuated by its originator in order to streamline the formulation of a host of data on enzymatic equilibria. This basic orientation has repeatedly proved most fruitful. The understanding for instance of the

role of adenosinetriphosphate (ATP) in biological acetylations would hardly be possible without a vigorous and bold attempt to grasp some of the physico-chemical characteristics of bond exchanges.

A few words about the terminology used in this review are necessary. Enzymic and isotope techniques have assisted us in locating the linkage which is the target of "attack" in enzymic phosphorylations. In order to facilitate the understanding and formulation of the many intricate exchange reactions, the authors have found it useful to try to classify according to the anhydro group which is supposed to be transferred. The classification is primarily for the purpose of posing and exposing more or less presupposed chemical concepts in enzymology.

Phosphorylase, transglycosidases, and purely hydrolytic glycosidases are all to be classified as enzyme-transferring "glycosyl" [cf. Hoffmann-Ostenhof (123)], i.e., a transfer to phosphate, to the hydroxyl group of an organic compound, or to water. Likewise, the Kornberg pyrophosphorylase is classified as adenyl transferase [the adenyl going between pyrophosphate and N^+ -(nicotine-amide)-ribose-5-phosphate (166)]. These considerations are of more than academic interest as the following example will show. It has been found that in the synthesis of active CoA an enzyme seems to be "charged" by ATP with the formation of a supposed "adenyl" enzyme and free pyrophosphate (143). In the present review this process would be classified as an "adenyl" transferase, catalyzing the transfer of this "anhydro" group between inorganic pyrophosphate and an X group in the enzyme. More recently it has been reported (204) that in the synthesis of a certain peptide bond the enzyme catalyzing this process seems first to be "charged" with a pyrophosphoryl group, leaving free 5-adenylate in solution. This would be a true pyrophosphorylation and this enzyme should not be called pyrophosphorylase. Pyrophosphoryl transferase seems a logical and proper term as does adenyl transferase.

Since the term "kinase" for the phosphorylation of hydroxyl groups by ATP was introduced about 30 years ago and has been retained, it will be used also in this review. In a recent review [Hoffman-Ostendorf (123)] the name "transphosphatase" has been suggested. Since the ATP system here presumably functions as a "phosphoryl" donor, the name "phosphoryl transferases" might be more appropriate. However, since the reaction mechanism for this class of enzyme is not always too obvious, it has been thought advisable to keep the old term "kinase."

THE INVOLVEMENT OF ADENOSINE TRIPHOSPHATE AS PHOSPHORYL DONOR

Burton & Krebs (43a) have recalculated the pre-energy changes associated with a hydrolysis of the pyrophosphate linkage between the γ (terminal) and the β phosphorus. At pH 7.5 and 1 M activity, ΔF of hydrolysis was found to be -9.4 kcal. For the concentrations of ATP in animal tissues the values would range between -13 and -16 kcal. In transphosphorylations or

related reactions the absolute concentrations are of minor importance. For equimolar amounts of phosphoryl donor and acceptor the standard values would essentially be valid.

PHOSPHORYLATION OF NUCLEOTIDES (REVERSIBLE KINASES)

Phosphorylation of 5-monophosphorylated purine nucleosides.—Leuthardt & Bruttin (195) found that the soluble part of liver homogenates contains an adenylic acid kinase which is stable to heating in 0.1 *N* HCl. The reaction products were separated by ion exchange using Dowex 1. Adenylic acid kinase has been prepared by Gilmour & Calaby (101) from insect muscles by the procedure of Colowick & Kalckar for the preparation of the enzyme from rabbit muscles. Kitiyakara & Harman (159) found myokinase of pigeon breast muscles to be associated with the mitochondria. The enzyme of mitochondria has been studied by Siekevitz & Potter (281), using ion exchange chromatography for separating the reaction products. The Michaelis constant for adenosine diphosphate was found to be 1.3×10^{-3} *M*. The enzyme was completely inhibited by 0.01 *M* fluoride and was somewhat activated by magnesium ions. The specific activities of mitochondria of fed rat liver, regenerating rat liver, and of fasted rat liver were compared. Eggleston & Hems (84) determined the equilibrium constant of the adenylate kinase system by using paper chromatography for separating the reaction components. They found $[(\text{ATP}(\text{AMP}))]/(\text{ADP})^2 = 0.444$. This is confirmed by other investigators [Green, Brown & Mommaerts (107a)]. Cobey & Handler (60) measured the time course of the specific activity of each of the phosphates in ATP in whole organs after administration of P^{32} labelled inorganic phosphate. From their results they concluded that *in vivo* the adenylate kinase reaction is much more rapid in kidney and liver than in muscle. The specific activity of the β -phosphate of ATP is taken as an indirect measure of the AMP^2 concentration in the organ. The adenylate kinase reaction in kidney liver proceeds at almost the same rate as the turnover of the γ -phosphate of ATP. Ashwell & Hickman (9) found a heat labile 5-adenylate kinase in spleen homogenate. 5-Adenylate kinase activity as measured spectrophotometrically in the presence of magnesium ions and myosin B can be removed from the latter by dissolving and precipitating the myosin B several times [Bowen & Kerwin (32)].

Dounce & Kay (79) report that adenylate kinase is able to catalyze a phosphorylation of ribonucleic acid (RNA^2). Dounce has proposed a very suggestive hypothesis (79) in which the bound 5-phosphorylated nucleosides of RNA are supposed to act like free 5-adenylate as a phosphate acceptor for ATP catalyzed by adenylate kinase. The authors find a distinct increase in total P of the incubated RNA if adenylate kinase and ATP both are present. In order to obtain more proof, it is necessary to isolate the supposed pyrophosphonucleotides (Dowex 1 chloride chromatography should be very suitable). It is at least necessary to demonstrate the presence of ATP labelled phosphorus in the nucleic acid before this highly interesting suggestion can

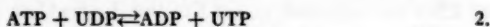
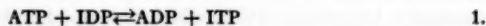
be taken more seriously. The formation and especially accumulation of a tri-ester [cf. also Ronwin (268)] would offhand seem unlikely, but perhaps secondary reactions take place which will make the isolation of pyrophosphates very difficult. The speculative theory (78) of a polyacylphosphorylated polymer as a suitable center for reproduction of polypeptides [cf. analogous pattern proposed by Lipmann (204)], nucleic acids, or polymetaphosphates is intriguing and may well stimulate progress.

Krebs *et al.* (176a) found that in suspensions of rat liver and pigeon breast muscle, the two labile phosphate groups of ATP, can react at equal rate which is probably accounted for by the presence of adenylate kinase. In the presence of boiled muscle extract or an ATP preparation of Roche Product Ltd. the pigeon muscle metabolized the β -phosphate of ATP markedly more slowly than the terminal phosphate (176a). In older studies on the same subject [Furchgott & Shorr (95); Flock & Bollmann (91)], it was found that the terminal group of ATP reacted markedly faster than that of the β -phosphate, whereas in studies with rabbits [Kalckar *et al.* (148)], it was found that *in vivo* the two groups had attained the same isotope concentration a short time after the injection of the radioactive phosphate. In the frog it was even possible to find a much higher isotope concentration in the β -phosphate than in that of the α in cases where the creatine phosphate had a markedly lower P^{32} concentration than that of the average acid-labile P of ATP [Kalckar *et al.* (148)].

Slater (286) finds strong evidence against 5-AMP being phosphorylated through oxidations in the heart muscle sacrosomes like ADP; in their opinion, adenylate kinase is the only enzyme catalyzing phosphorylation of AMP.

The enzyme, 2-amino-adenylate kinase (which may be identical with adenylate kinase since they have many properties in common, such as stability towards heat and acid), occurs in yeast, together with the corresponding nucleoside kinase [Kornberg & Pricer (168)]. If phosphopyruvate is used as the ultimate phosphate donor (and in the presence of the proper phosphoryl transferases and the ADP as a transfer system), 2-amino adenosinetriphosphate can be obtained in good yield. If ATP can phosphorylate 2-amino adenosinediphosphate to the corresponding triphosphate, this step would be catalyzed by an enzyme to be classified in the next section.

Nucleoside diphospho kinases ("Nudiki").—Berg & Joklik (19) have isolated and purified an enzyme from yeast which catalyzes the following reactions:



in which ITP² and UTP signify inosinic- and uridytriphosphates respectively. 5-AMP, 5-IMP, 5-UMP, or creatine do not react.

The reaction has been found to be necessary for the participation of ITP and UTP in the phosphorylation of creatine and glucose. Using ITP or UTP with corresponding enzymes gives no reaction or an insignificant one

until a catalytic amount of ADP is added (20). Likewise the analogous triphosphate has only an effect on the actomyosin concentration phenomenon, again with a catalytic but even smaller amount of otherwise inactive ATP.

ATP or ITP were labelled with P^{32} in the beta- and gamma-positions. ATP, ITP, ADP, and IDP were separated by paper ionophoresis at pH 3.5 in $\mu/50$ citrate buffer. Reaction 1 could be a result of a transamination [cf. Muntz (236)]. To distinguish between these two possibilities each of the four components was isolated, and the results obtained were compared with the values predicted by each type of mechanism. Table I summarizes the results.

TABLE I
THEORETICAL AND EXPERIMENTAL VALUES FOR ATP-IDP
ENZYME-CATALYZED REACTION*

Compound Isolated	Theoretical Values For:		Values Found:
	Transamination	Transphosphorylation	
ATP	1920	1920	1920
IDP	0	0	0
ADP	0	960	1045
ITP	1920	960	930

* Summarized from Berg & Joklik (19).

The results in this table unambiguously establish that reaction 1, catalyzed by the yeast enzyme, is a transphosphorylation and not a transamination. Although the enzyme is acid-stable, it is not identical with adenylate kinase. The equilibria of reactions 1 and 2 are both close to one indicating that the ΔF of hydrolysis of the pyrophosphate bonds of ITP are about the same as that of ATP.

Krebs & Hems (176) have probably encountered the same enzyme in their studies of nucleotide metabolism in muscle and intestinal mucosa, although a transamination is not excluded here. They find that the transfer of P^{32} from ATP (Adenosine P— P^{32}) to IDP is not catalyzed by any of the adenosinetriphosphatases which are known to catalyze a transfer to hydroxyl groups.

It should finally be added that the observation that ATP+UDP in the presence of yeast juice formed Co *Galacto* *Waldenase* [Trucco (304)] already inferred the existence of "nudiki."

FORMATION OF PHOSPHO-AMIDINES

The creatine kinase which catalyzed the equilibrium ATP+creatine and ADP+phosphocreatine has been studied in relation to the properties of actin by Feuer & Wollemann (88, 329). The creatine kinase of muscle is

mainly (ca. 80 per cent) obtained as a water soluble enzyme. However, about 20 per cent of the enzyme was found to be strongly bound to actin. Although the soluble and the actin-bound enzymes have the same isoelectric point (pH 4.6), only the latter precipitates out at this pH. The pH optimum as well as the effect of pH on the equilibrium is the same for both enzymes. The latter factor is greatly influenced by cations. At pH 6.7 K^+ favors formation of phosphocreatine, as does Ca^{++} at pH 7.2, whereas at the higher pH, K^+ favors ATP formation. Actin from rabbit muscle contains creatine kinase and some free creatine (ca. 0.03 per cent). Removal of creatine kinase from actin deprives the latter of the ability to polymerize by addition of various salts. Addition of the creatine kinase to the heated actin restores the ability to polymerize [cf. also earlier work by Laki *et al.* (183)]. Goodal & Szent-Györgyi (105) found that fibers from glycerol treated muscle could be brought to relaxation by addition of creatine kinase and phosphocreatine.

Lorand (209) has added to the proof that creatine kinase is an important factor in the relaxation phenomenon. The reaction is formulated as follows: $(ADP)AM + P \rightleftharpoons (ATP)AM + \text{creatine}$, in which " $(ATP)AM$ " signifies a supposed combination of the nucleotides with actomyosin. The possibility is discussed that the relaxing factors of Bendall (16) work in a similar fashion. For further information the reader is referred to the chapter on "Chemistry of Muscle" by Mommaerts. Creatine phosphokinase has been studied in unfractionated brain extracts by Narayanaswami (240). This enzyme reaction was somewhat faster with AMP than with ADP as phosphate acceptor. The pH optimum for phosphorylation of AMP was between 6 and 7, whereas for the phosphorylation of creatine by ATP it was found to be above 8.2. Of a number of compounds tested only iodoacetate, and to a lesser extent fluoride, was inhibitory. Kuby *et al.* (179) has purified the creatine phosphorylating enzyme from rabbit muscle by heat denaturation and repeated alcohol precipitation of the protein-magnesium salt. Witter *et al.* (328) found no effect of DNP on phosphocreatine or phosphopyruvate transphosphorylase. Kitiyakara & Harman (159) found creatine phosphokinase to be present predominantly in the soluble part of pigeon breast muscles.

Tseitlin (307) described an enzyme from rat brain which catalyzes a dephosphorylation of phosphocreatine. Unlike the system in muscle, it does not seem to go through the ADP-ATP system. The hydrolysis of phosphocreatine proceeds at a high rate in the absence of adenynucleotides in the dialyzed extracts, and if ATP is added to creatine in the presence of the brain extract, only small amounts of phosphocreatine are formed as compared with muscle. Since the incubation periods were short and the adenosinetriphosphatase activity only moderate, there would have been sufficient ATP present during the incubation period to yield an extensive phosphocreatine synthesis if the transfer system had been operating. The enzyme is apparently present only in small amounts.

FORMATION OF ACYL PHOSPHATE

The phosphorylation of a carboxylate group by ATP was described first in Warburg's laboratory [Bücher (43)]. The acylphosphate formed, 3-phosphoglyceryl phosphate, was shown to rephosphorylate ADP in the presence of a highly active phosphoryl transferase. The enzyme is widespread in plants [Stumpf (298)]. It is inhibited by fluoride but is insensitive to high concentrations of hydroxylamine. Axelrod & Bandurski (9a) have recently obtained the hydroxamic complex of 3-phosphoglyceric acid from 3-phosphoglyceraldehyde.

The formation of 3-phosphoglyceryl phosphate (Warburg-Negelein ester) from 3-phosphoglyceraldehyde.—The formation seems to follow the pattern first suggested by Lynen & Racker, i.e., formation of a mercaptal complex which is oxidized through DPN² to an acyl-mercapto compound which subsequently undergoes phosphorylation. This topic is reviewed in detail elsewhere (5), and therefore only a few pertinent points will be discussed here. According to the formulations by Velick & Hayes (311) and by Segal & Boyer (280), the equilibrium constant, K' , for the over-all oxidative phosphorylation is the product of two constants K_1' and K_2' which signify the following:

$$K' = K_1' \times K_2' = \frac{\begin{array}{c} \text{O} \\ \parallel \\ (\text{R}-\text{C}-\text{Enz}) \end{array} (\text{DPNH}) (\text{H}^+)}{\begin{array}{c} (\text{RC}-\text{H}) \\ \parallel \\ \text{O} \end{array} (\text{Enz}) (\text{DPN}^+)} \times \frac{\begin{array}{c} \text{O} \\ \parallel \\ (\text{R}-\text{C}-\text{O}-\text{PO}_3^{--}) (\text{Enz}) \end{array}}{\begin{array}{c} (\text{RC}-\text{Enz}) \\ \parallel \\ \text{O} \end{array} (\text{HPO}_4^{--})}$$

K' was determined to be 1×10^{-7} (226), and K_1' to be 10^{-5} (311) and therefore $K_2' = K'/K_1' = 10^{-2}$. This value is close to that of the corresponding acetyl-phosphate and CoA interaction (294). Harting (112a) found three years ago that this enzyme can catalyze acetyl-phosphate formation.

In the case of the lower or higher fatty acids or the dicarboxylic acids, acyl compounds are formed with the mercapto groups of thioctic acids or CoA yielding acyl mercapto linkages. It seems clear now that oxidative phosphorylation (including anaerobic "oxidations") can be brought about by a phosphorolytic cleavage of an acylmercapto linkage (which again is the result of an oxidation of a mercaptal) yielding a free sulphydryl group and acylphosphate. The latter can in turn phosphorylate ADP to ATP. The phosphorolytic fission of an acyl CoA was discovered first for acetyl CoA [Stadtman (294)] and called phosphotransacetylase (acetyltransferase) since the anhydride "acetyl" was transferred from CoA to phosphate. Whiteley (324) found indications that succinylphosphate is formed in propionic acid bacteria probably through a "CoA transferase." [Hift *et al.* (120) and Kaufman (154) found this acylphosphate in mammalian tissues.

Heart muscle contains an enzyme system which in the presence of succinyl CoA stimulated the phosphorylation of ADP [Hift *et al.* (120)]. This

was taken as one indication for an intermediary formation of succinyl hydroxamic acid [Green *et al.* (106); Drysdale & Lardy (81)]. In microorganisms acetylphosphate can act as a phosphoryl donor for ADP [Lipmann (203); Korey (quoted in 294a)] as well as an acetyl donor for CoA [Stadtman (294)]. The latter faculty is of importance for acetoacetate synthesis, also in animal tissues [Lynen & Ochoa (211); Green *et al.* (106)]. Recently Stadtman & White (294b) found an enzyme system from *Clostridium kluyveri* which catalyzes the acetylation of imidazole by acetyl phosphate. They resolved the reaction into an acetylation of CoA by acetyl phosphate (phosphotrans-acetylase) and a subsequent acetylation of a nitrogen of the imidazole by acetyl-S-CoA. Finally the N-acetyl imidazole can acetylate cysteine non-enzymatically, and independent of CoA, to N-acetyl-cysteine. Bessman & Lipmann (25) have found enzymes in animal tissue which can catalyze a transacetylation from one arylamine to another, presumably through an "acetyl-enzyme." The same enzyme preparation is able to catalyze acetylation of aromatic amines from acetyl CoA. An interesting novel variation of this reaction which can be demonstrated to occur in both directions is the formation of β -aspartyl phosphate from ATP and a yeast enzyme [Black & Gray (26)]. The equilibrium is unfavorable towards the formation of the acetylphosphate, but hydroxylamine can be used as a trap forming the corresponding hydroxamic acid [Lipmann & Tuttle (202)]. Enzymic "trapping" of ADP (i.e., by addition of adenylate kinase and deaminase) should improve the yield of β -aspartyl phosphate. The reaction which is a typical reversible transfer of "phosphoryl" is specific for L-aspartate. This is the first acyl phosphate in the amino acid series to be discovered. Since it is a β -acyl compound, it might presumably be an intermediary in peptide formation as well as in asparagine formation. In the latter case aspartyl phosphate should serve as an aspartyl donor and ammonia as acceptor (ammonia aspartyl transferase), and α -phosphate would be liberated. Most recently Black & Wright (27) have shown that β -aspartyl phosphate can undergo a reduction of the acyl group thus converting the aspartyl skeleton into a homoserine skeleton [see review on oxidation reductions in this volume (7)].

Although ATP plays an important role in the "activation" of fatty acids, dicarboxylic acids, and certain amino acids, it is only in a few instances, and then mainly in microorganisms, that a formation of acyl phosphate either by acyl transferase or by phosphoryl transferase, takes place.

Kornberg & Pricer (170) have demonstrated the enzymic formation of the acyl CoA compounds of higher fatty acids. The palmityl CoA compound was isolated and purified. The enzyme system was derived from guinea pig liver. In the presence of hydroxylamine the corresponding hydroxamic acid is formed, and ATP is broken down to 5-adenylic acid and inorganic pyrophosphate. In the presence of CoA in stoichiometric amounts the balance is the same except that acyl CoA accumulates. The isolated palmityl CoA (and synthetically produced palmityl CoA) in the presence of α -glycerophosphate (P^{32} -labelled) and a liver enzyme (but in the absence of ATP and

CoA) gave diacyl (palmityl) phosphatidic acid which was isolated and identified. If free palmitic acid is used, catalytical amounts of CoA and stoichiometric amounts of ATP have to be added. For each mole glycerophosphate incorporated two moles of ATP were split to 5-adenylic acid and pyrophosphate. In some most elegant experiments in which the palmitic acid was labelled with C^{14} and the glycerophosphate with P^{32} , the purified product was shown to contain two moles of C^{14} -esterified material for each mole of P^{32} material. This represents great progress in our understanding of the biosynthetic pathways of phospholipid synthesis.

In a large number of interesting biosynthetic step reactions, the role of ATP is more or less concealed. It is especially in these cases that the use of isotopes has provided us with new ideas.

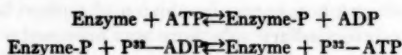
FORMATION OF PHOSPHORYL (OR "R-PHOSPHORYL") ENZYMES

As mentioned in the introduction, later studies on labelled esters have encouraged the idea that the anhydrous partner of an ester forms a covalent bond with a group in an enzyme. This hypothesis would explain the observations that ATP is necessary in many coupled syntheses and is consumed strictly stoichiometrically in a seemingly hydrolytic splitting. A speculation on myosin adenosinetriphosphatase in which a phosphorylation of the myosin occurred as an intermediary substance was proposed a number of years ago (147, 264). In the systems discussed here, we are dealing with a purely chemical coupling, and there should be a more rational basis for finding indication of such enzyme intermediaries [phosphoryl-enzyme, pyrophosphoryl-enzyme, nucleotidyl enzyme (see later sections)]. The presence of enzyme, the appropriate organic acid, CoA, and, in addition, ATP are required in the formation of the following: (a) acetyl-CoA (203), (b) acetoacetyl CoA (295), (c) β -hydroxybutyryl CoA (187), (d) butyryl CoA (316), (e) succinyl CoA (154). In (a) and (b) inorganic pyrophosphate is liberated [Jones *et al.* (143); Beinert *et al.* (14); Stern *et al.* (295); Lehninger & Greville (187)], whereas in (c) one mole orthophosphate was liberated for each mole succinyl CoA formed [Kaufman (154)]. Reaction (b) is strongly inhibited by inorganic pyrophosphate. This is of great interest in understanding the mechanism whereby ATP acts as a phosphoryl donor.

It has been known for some time that ATP is necessary for synthesis of peptide "linkages" as well as for the formation of amides. Glutathione (GSH^2) synthesis in extracts from acetone-dried liver, from glutamate, cysteine, and glycine, or from glutamyl cysteine and glycine is greatly enhanced by addition of phosphorylated intermediaries of glycolysis (hexose-, mono-, or diphosphate, phosphoglycerate, phosphopyruvate) in addition to ATP and inorganic phosphate. It is peculiar that in dialyzed fractions 5-adenylic acid is more active than ATP [Bloch *et al.* (142, 289, 334)]; in purified fractions ATP proves to be the most active.

In a more purified fraction in which adenosinetriphosphatase activity is practically eliminated, ATP when supplemented with phosphoglycerate

still brings about a distinct enhancement, although much smaller than that observed in the cruder fractions. This is ascribed to the fact that the accumulated ADP (adenylate kinase absent) which exerts an inhibitory effect on the GSH synthesis is continuously being rephosphorylated to ATP. The enzyme which catalyzes the condensation of γ -glutamyl cysteine and glycine to GSH has been purified 50-fold from extracts of acetone-dried liver (290). The synthesis, which has an absolute requirement for ATP and Mg^{++} , is stimulated markedly by K^+ . One mole of terminal P from ATP is split for each peptide linkage formed. It was argued that this phosphate did not originate secondarily from liberated inorganic pyrophosphate. Most recently, Snoke (291) has reported observations which may have close bearing on the mechanism of ATP in this synthesis. It was shown that for each mole of glutamine formed, one mole of ATP was split into ADP and P. There was no change in the amount of 5-AMP. If P^{32} -labelled ADP ($AR-O-P-O-P^{32}$) was added to highly purified enzyme together with unlabelled ATP, after a short incubation time it was found that the ATP had exchanged very markedly with the ADP; the P^{32} concentration after 1 hr. at $37^\circ C.$, using $3\mu g.$ enzyme per ml., was found to be equal in both nucleotides. The author suggests the following formulation:



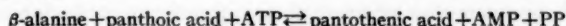
These equations may also explain the peculiar inhibition by ADP on the GSH synthesis. The formation of P^{32} labelled ATP may be attributable to adenylate kinase. However, the fact that $3\mu g.$ of enzyme gave a 92 per cent exchange and $30\mu g.$ of the enzyme 99 per cent in the same period of time, is a strong indication against adenylate kinase. The latter would have yielded: $AR-O-P-O-P^{32}-O-P^{32}$, i.e., a nucleotide with the double amount of labelled P per mole nucleotide. If this reaction had taken place, ATP with P^{32} exclusively in the β -phosphate (i.e., $AR-O-P-O-P^{32}-O-P$) would be formed. This has not yet been demonstrated but should be easy to do. This system is probably an example of a series of step reactions in which phosphoryl enzyme is formed as an intermediate. O^{18} labelled water will definitely establish whether this reaction proceeds with a fission between the oxygen and the terminal phosphorus forming "phosphoryl" and ADP, the former phosphorylating a group in the enzyme. The relation of nucleoside-diphosphokinase (19, 176) to this system deserve attention.

In the closely related enzyme system which catalyzes the formation of glutamine in the presence of ATP, glutamate, and ammonia [Speck (293); Elliott (85)] a closely related mechanism may be operating. Elliott has recently purified the system to a high degree, and Levintow & Meister (198) have studied the rate of synthesis of the D- as compared with the L-glutamine. Interestingly, in the formation of the hydroxamine compound, a reaction which also requires ATP, the D and L form react equally fast and at high rates. If ammonia is added instead of hydroxylamine, L- and D-gluta-

mine is formed. However, in this case the L form reacts at least twice as fast as the D form. Thus, in what might be considered the process of making "active" glutamate by enzyme and ATP (presumably through a reaction between enzyme and ATP) there is no discrimination between D- and L-forms. Only in the reaction between the supposedly active glutamates and ammonia does the discrimination appear. The detailed mechanism of ATP activation in this system is obviously of great interest. In the enzymic synthesis of arginine through a condensation product between citrulline and aspartic acid (258, 259), it has been shown that ATP is a component and that close to two moles of labile P are liberated for each mole of citrulline converted to arginine [Ratner *et al.* (260)]. In the formation of citrulline from carbonylglutamate, ammonia, and carbon dioxide, ATP is a participant, and a compound X is presumably formed as a precursor to citrulline [Grisolia & Cohen (110)].

In neither of these cases has it been possible to detect formation of " $\sim P$ " compounds and it is therefore not unlikely that a " $\sim P$ " compound or related linkage is formed with one of the enzymes.

Pantothenate synthesis.—In a study of the ATP-requiring enzyme reaction for the synthesis of pantothenate with extracts from *Escherichia coli*, Maas has found (212) that the reaction involves a splitting of ATP with the liberation of pyrophosphate and that the over-all reaction proceeds in the following way:



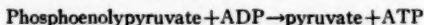
CoA seems not to be involved in the reaction and ADP does not substitute for ATP as energy source. It has recently been observed [cf. Lipmann (204, 213, 214)] that the formation of the peptide linkage between panthoate and β -alanine seems to be coupled with a pyrophosphorylation of the enzyme protein. This appears from the fact that radioactive adenylate (and not pyrophosphate) can exchange with the adenyl residue of ATP in the absence of the carboxyl partner (panthoate). In the presence of the latter pyrophosphate also can exchange with the pyrophosphate of ATP. Addition of the carboxyl acceptor, β -alanine brings about the formation of the second peptide linkage of pantothenic acid and the enzyme group is supposed to be "free" again. Jones *et al.* (144) have found another type of "activation" of an enzyme group in this case with the nucleotidyl part. This interesting case will be described under the Nucleotidyl Transferases Section.

PHOSPHOPYRUVATE FORMATION FROM ITP AND ATP AND OXALOACETATE

A system which must be classified completely separately is the reaction by which phosphopyruvate is formed from dicarboxylic acid. It was found some 15 years ago that dialyzed homogenates from kidney in the presence of fluoride form phosphoenolpyruvate, if malate or fumarate is added together with inorganic phosphate (146a). At that time the reaction was for-

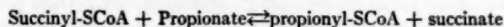
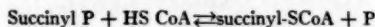
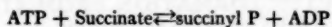
mulated as phosphorylation of oxaloacetate by ATP with a subsequent decarboxylation. This formulation has been proved to be partly correct by the recent experiment by Utter & Kurahashi (310). Utter & Kurahashi have found a most interesting aspect, that the reaction as catalyzed by an enzyme from pigeon liver can be reversed. The conversion of the phosphoenol linkage into a pyrophosphate linkage in ATP or ITP, a reaction which under most physiological conditions would release about 4 kcal. per mole, is coupled with an incorporation of carbon dioxide as a carboxyl group. Whether phospho-oxaloacetate really is formed as an intermediary is unknown and perhaps even doubtful. It is also of great interest that the ionosine pyrophosphates were found markedly superior to the corresponding adenosine pyrophosphates in the reaction. The mechanism of this rather complex but amazingly efficient synthesis will undoubtedly be clarified by further studies, and the result will be anticipated with the greatest interest. Bandurski & Greiner (12, cf. also 12a) have found an enzyme in spinach leaves which catalyzes the carboxylation of phosphoenolpyruvate yielding oxaloacetate and inorganic phosphate. Pyruvate+ATP cannot replace phosphopyruvate. Addition of ADP did not increase the rate of reaction; however, this may be a result of recycling of trace amounts of ADP or IDP. Interesting relations between photosynthesis and ATP synthesis were found by Goodman *et al.* (105a) which may be pertinent to Utter & Kurahashi's work.

Pyruvate phosphoferase.—A study of the interaction of certain cations and of phosphopyruvate with pyruvate phosphotransferase catalyzing:



has been made by Kachmar & Boyer (145). The enzyme has an absolute requirement for potassium, ammonium, or rubidium ions, for all of which the Michaelis constant is about 0.011 *M*. In the case of K^+ this figure was independent of the concentration of phosphopyruvate, as the Michaelis constant for phosphopyruvate (8.6×10^{-4} *M*) was independent of the concentration of K^+ . Na^+ and Li^+ counteracted the activating effect of K^+ . Ca^{++} inhibited the reaction in a rather atypical way. From the kinetics of the reaction the authors concluded that an active ternary complex is formed by independent combination of K^+ and phosphopyruvate with enzyme.

Whitley (324) has found that extracts of *Micrococcus lactilyticus* decarboxylates succinate to propionate. This decarboxylation requires ATP, CoA, and cocarboxylase. From the distribution of C^{14} and other data the author suggests the following sequence of reaction:



The latter reaction is actually a transfer reaction of the CoA—S group. The possibility of a transfer of "active" CO_2 is briefly discussed.

Bowen & Kerwin (32) report that the P^{32} is distributed to different places

depending on whether the plant is exposed to light or not. In short dark experiments the most P^{32} was found in ATP. In short light experiments a larger proportion of P^{32} was found in phosphoglycerate.

Several investigators [Slade (284); Oginsky & Gehrig (245); Knivett (163)] have found that cell-free extracts of *Streptococcus faecalis* convert citrullines to ornithine, CO_2 , and ammonia, a reaction which is accompanied by phosphorylation of ADP to ATP. Radioactive inorganic phosphate was used in order to distinguish between the ATP formed from ADP and P, and the ATP formed from ADP by adenylate kinase. The latter would not give rise to occurrence of P^{32} in ATP. Initially there was a formation of ATP from ADP approximately equivalent with the CO_2 formation from arginine. It would be interesting to see whether this system would convert IDP to ATP, i.e., is there a transamination from one amide to another? The system described by Ratner & Petrack (258, 259) is also of interest in this connection. As mentioned previously in the oxaloacetate decarboxylation system, ITP is superior to ATP. The carboxylation of ornithine is greatly enhanced by addition of N-acyl glutamic acid compounds such as N-carbonyl, N-acetyl, and N-formyl glutamate. [Grisolia & Cohen (110)].

Other synthetic ATP-requiring reactions.—Some of the ATP-requiring synthetic enzyme reactions for which the mechanism is entirely unknown will briefly be mentioned. Under anaerobic conditions the enzymatic conjugation of *m*-aminophenol with sulfate is entirely dependent on ATP [Bernstein & McGilvery (22); De Meio *et al.* (74)]. Additional experiments of Bernstein & McGilvery (23) suggest that it is sulfate which is activated by ATP.

Further studies by Cantoni (47) on the activation of methionine by ATP catalyzed by enzyme from rabbit liver for the formation of 5-adenosyl methionine suggest that several enzymes are involved. Our understanding of the mechanism of this interesting reaction, although still obscure, has been greatly advanced by these studies. It seems most likely that a kind of thioelastic fission of ATP takes place by which the carbon oxygen linkage of the C_5 of the ribose of adenosine is attacked. The adenosine part is incorporated in a special linkage involving the formation of a sulfonium compound ("active methionine"). The fate of the phosphates of ATP is conversion ultimately to three moles of orthophosphate. The following findings support the hypothesis that active methionine is S^+ -adenosyl methionine [methyl (5-deoxyriboseyl-adenine)-(2-aminobutyro)thetin]. If the rabbit liver enzymes are incubated with ATP and S^{35} -labelled methionine, an adenosine spot on the paper chromatogram containing S^{35} can be detected. This could be thio-methyladenosine (cf. 276) or a derivative of this compound. More important was the fact that when the added methionine was labelled with C^{14} in the C_2 carbon, the same adenosine spot contained C^{14} , i.e., the entire methionine carbon skeleton seems to be present in the adenosine spot. Moreover, an eluate of the spot proved to be an active methyl donor for guanidoacetic acid in the presence of the proper methyltransferase. The work of Borsook & Dubnoff (30) showed that enzyme methylation of guanidoacetic acid to creatine de-

pended not only on the presence of methionine but also on ATP. Upon hydrolysis a substance was liberated which if subjected to chromatography migrated like homoserine. The presence of a sulfonium (S^+) compound also indicates that the "adenosine" spot migrates as a cation by paper ionophoresis at pH 7 to 8. Inorganic triphosphate is apparently not formed, but recent findings by Cantoni (48) indicate that first the terminal (γ) phosphate may be liberated as orthophosphate perhaps by a thioclastic reaction, and subsequently the α - and β -phosphates are liberated as inorganic pyrophosphate concomitant with an attack of the carbon oxygen linkage of the 5-ester phosphate by the methylsulfonium group. Cantoni has suggested that perhaps a thioclastic cleavage of the terminal phosphate follows a pattern like toluene sulfonyl adenosine in which the acid group in the 5-position reacts with one of the nitrogens of the adenine [Clark, Todd & Zussmann (58)]. The synthesis of creatine from guanidoacetic acid and methionine in the presence of ATP by whole homogenates of liver has been studied by Cohen (61). The properties of the system from different animals were investigated with regard to optimum conditions for synthesis.

The role of ATP in many other reactions such as biological incorporation of formyl groups into purine precursors [Greenberg (108)] will undoubtedly be a fertile field for further research.

DEAMINATION OF 5-NUCLEOTIDES

In general it should be borne in mind that the amino groups found in purines like adenine or guanine should be classified closer to amidine (imidine) groups than to amino groups. The "deamination" is irreversible in general as is the "deamination" of arginine to citrulline, a type of reaction to which it may be related and correlated.

Webster (322) as well as Deutsch & Nilsson (75) report evidence for a direct deamination of ADP to IDP. A compound is formed which gives a chromatographic spot of about the same R_F as authentic IDP. Muntz (237) describes an enzyme preparation from dog brain which catalyzes the deamination of 5-adenylic acid. In contrast to Schmidt's 5-adenylic acid deaminase from muscle, the brain enzyme system requires the addition of ATP. In one way ATP is required in stoichiometric amounts, for maximum rate of deamination. In another sense ATP acts catalytically since repeated additions of 5-adenylic acid bring about rapid deamination without repeated addition of ATP. ATP is not consumed in the final balance. However, Muntz discusses the possibility of a primary deamination of ATP to ITP which is subsequently reaminated by 5-adenylic acid, the latter being converted to 5-inosinic acid.

ATP AND BIOLUMINESCENCE

The mode of action of ATP on the luminescence in fireflies extract still remains unknown; however, the recent discovery that luminescence in extract from *Achromobacter fischeri* (which do not require ATP for the reaction)

requires long-chain fatty aldehydes (plasmas) [Cormier & Strehler (66)] raises the possibility of formation of an intermediary acyl enzyme complex. The fatty aldehydes are active only provided the aldehyde group is free. This suggests the formation of a mercaptal which subsequently undergoes oxidation. There may be cases analogous to the phosphoglyceraldehyde dehydrogenase system in the case of bacterial luminescence and analogous to phosphoglyceryl-P formation with ATP as phosphoryl donor (or the phospho transacylase system) in the case of firefly bioluminescence.

McElroy and his group (221, 222) have continued studies on the effect of inorganic pyrophosphate on the luminescence of firefly extracts. They had found an inactive complex of luciferase subsequent to mixing luciferase and luciferine with ATP and Mg^{++} . The inactive complex manifests itself by a rapid decrease in light intensity after mixing the above-mentioned components. The low base line level of luminescence is presumably a measure of a steady state between the inactive and the active complex.

The formation of the inactive complex depends not only on the presence of ATP and Mg^{++} , but also on the presence of a second protein which may be identical with inorganic pyrophosphatase. Thus, purified luciferase preparations which do not catalyze the hydrolysis of pyrophosphate show a high steady state level of luminescence. Addition of pyrophosphate or triphosphate after the initiating reaction with ATP greatly stimulates light production presumably by decomposing the inactive complex ("secondary luminescence"). Inhibitors of inorganic pyrophosphatase (Mn^{++} , Ca^{++} , F^{-}) prevent a rapid decay of luminescence, if the secondary luminescence was brought about by extra addition of pyrophosphate. Since triphosphate is not hydrolyzed by any of the enzyme preparations, the steady state of luminescence remains at a higher intensity after addition of the compound, and the above mentioned ions have no further effect. If pyrophosphate or triphosphate are added together with Mg^{++} to luciferase prior to the addition of ATP, the light response to the latter is greatly suppressed. Apparently the inorganic pyrophosphates, as well as ATP, compete for the active as well as the inactive complex. That luciferase itself is complex is borne out by the fact that successive frequent additions of the enzyme elicit repeatedly small responses succeeded by a huge response upon addition of pyrophosphate. At low Mg^{++} concentration (2.5 to $5 \times 10^{-4} M$) 3 to 4×10^{-4} mole ATP yields optimum light response. It should, nevertheless, be stressed that it has not yet been possible to detect a hydrolysis of ATP coupled with bioluminescence. In general the previously mentioned observations indicating formation of pyrophosphoryl-enzyme or adenylyl-enzymes may also have bearing on the phenomena described here.

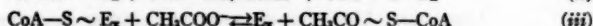
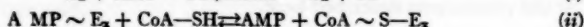
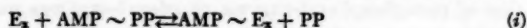
ADENOSINE MONOPHOSPHATES AS ENZYME ACTIVATORS

The monophosphorylated nucleosides seem in a few instances to be endowed with specific functions which are lost if the nucleotides are further phosphorylated. Thus, 5-adenylate (adenosine-5-monophosphate) is a specif-

ic activator of phosphorylase *b* [Cori & Cori (64)], and lately it has been found that 2-adenylate (adenosine-2-monophosphate) exerts an effect on TPN-DPN transhydrogenase from *Pseudomonas fluorescens* [Kaplan, Colowick & Neufeld (153)]. The seemingly irreversible reaction: $\text{TPNH} + \text{DPN} \rightarrow \text{TPN} + \text{DPNH}$ can be made reversible by addition of 2-adenylate. Two other derivatives, adenosine 2,5-diphosphate and adenosine 2-mono-5-diphosphate can replace 2-adenylate; whereas adenosine-3-monophosphate and adenosine-5-monophosphate are without affect. The 2-adenylate compounds also enhance the reaction in forward direction bringing about complete oxidation of TPNH^+ by DPN. Besides this reaction the analogous reactions between DPNH^+ and either deamino DPN or nicotinamide mononucleotide are also enhanced by the presence of 2-adenylate. The authors propose that 2-adenylate acts partly as a competitive antagonist of the inhibitory effects of TPN^+ (which is a 2-adenylate derivative) and partly as an activator. The activation site on the enzyme seems therefore to have specific requirements for the 2-adenylate structure.

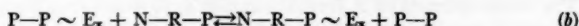
NUCLEOTIDYL TRANSFERASES

Adenyltransferases.—Since Kornberg's discovery of DPN and FAP² pyrophosphorylases (166) this type of reaction has been found to be important for several other biosynthetic reactions. Inasmuch as free pyrophosphate is an active component whereas the 5-adenylate part is active only as ATP, the reaction is actually a transfer of the adenylic anhydride ("adenyl") from inorganic pyrophosphate to another acceptor, like nicotinamide mononucleotide or flavin mononucleotide or groups which are part of protein molecules. We, therefore, propose to classify this type of enzymes as nucleotidyl transferases. Novelli and co-workers (243) have found that the peptide of β -mercapto ethanolamine with pantothenic acid after phosphorylation (terminal P or ATP) accepts (with the phosphate group as acceptor) adenyl from ATP forming the imminent precursor of CoA; the latter contains an extra phosphate group in the C₃ ribose. A more complex type of adenyl transferase has been described by Jones *et al.* (144). They found that P³² inorganic pyrophosphate exchanges rapidly with the pyrophosphate group of ATP in the presence of an enzyme from yeast which is involved in the synthesis of acetyl CoA. Addition of CoA suppresses the pyrophosphate exchange. If the E_x signifies the enzyme with an unknown acceptor group, PP a pyrophosphate group, AMP 5-adenylic acid anhydride, and CoA—SH the active acceptor group of CoA, the sequence of events was formulated as follows(144):



Step (i) may be classified as an adenyl transferase, Mg^{++} is needed; step (ii) is presumably not, since free adenylate is formed and apparently can react in the reverse direction, although the equilibrium favors formation of

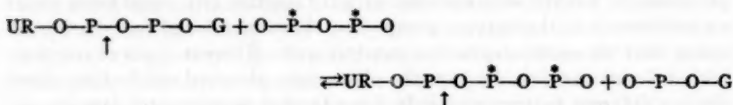
AMP and CoA—S—E_x. In (ii) the anhydro group is presumably on the enzyme. Studies with O¹⁸ labelled water should establish these points. If the phosphate in 5-AMP were enriched with O¹⁸ reaction (ii) would bring about an enrichment in the enzyme group. The above mentioned formulation assumes that the same enzyme can catalyze quite different types of reactions with different partners. Perhaps the phenomena observed can be formulated along a different pattern especially when further experimental data are obtained. Kornberg's DPN pyrophosphorylase is presumably acting like an adenyltransferase. However, since isotope exchange data are not as yet available other alternatives will have to be considered. E. R. Stadtman (personal discussion) pointed out that pyrophosphorylases might follow the three-step pattern of the acetate activation [Jones *et al.* (144)]. In that case Kornberg's reaction would not be a DPN pyrophosphorylase nor would it be an adenyl transferase, it would rather be a nicotinamide mononucleotidyl transferase. The sequence of reactions would be in this case:



where N—R—P signifies nicotinamide 5-phosphoriboside. This is important because exchange reactions with various groups of the substrate molecule should be tried. These considerations may also apply to the UDPG² pyrophosphorylase (or uridyl-pyrophosphate transferase). This possibility should be kept in mind when dealing with any transferase system.

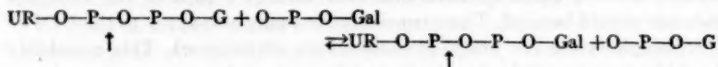
Uridyl transferases.—Addition of P³² labelled inorganic pyrophosphate to uridinediphosphoglucose (UDPG) has been observed in the presence of dialyzed uridinepolyphosphate compound. It could be adsorbed on norite and subsequently eluted with 50 per cent ethanol (234). The enzyme catalyzing this reaction was found to be abundant in "Zwischenferment" preparations from which it could be precipitated at pH 6 between 0.6 and 0.75 saturated ammonium sulfate at 2°C. The enzyme liberates Cori ester (in the presence of phosphoglucomutase, TPN, and glucose-6-phosphate dehydrogenase, 6-phosphogluconic acid and reduced TPN accumulate) and uridine triphosphate. Uridinetriphosphate, UTP, was separated from UDP and adenylpyrophosphates by paper chromatography [ethanol, ammonium acetate for 45 hr. (234)] or column chromatography using Dowex-1 chloride. It can be demonstrated and determined by a sensitive and quite specific enzymic assay. If UTP and α-glucose-1-phosphate are incubated with the same enzyme, UTP is consumed and UDPG is formed. If P³² labelled UTP was used, no radioactivity was found in the UDPG, all of it being liberated as free inorganic pyrophosphate. The enzyme is presumably acting like an uridyltransferase (more specifically uridyl-pyrophosphate transferase) according to the following formulation in which P* refers to P³², O—P—O—G to α-glucose-1-phosphate, UR—O—P—O—P—O—G to uridinediphosphoglu-

cose (uridinediphosphoglycosyl), $\text{O}-\text{P}-\text{O}-\text{P}-\text{O}$ to pyrophosphate, and $\text{UR}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}-\text{P}-\text{O}$ to uridinetriphosphate:



An entire glucose-1-phosphate unit is inserted and pyrophosphate is liberated.

In galactose-adapted *Saccharomyces fragilis* it can be shown that addition of UTP brings about a conversion of galactose-1-phosphate (174) to glucose phosphate (1 and 6-esters) in amounts which on a molar basis exceeds four to five-fold the amounts of UTP added (150). The observations made earlier by Trucco (304) that $\text{ATP} + \text{UDP}$ added to yeast juice operates like Co-galacto-Waldenase is probably, as he suggests, based on a combined action of "nudiki" [Berg & Joklik (19)] and uridyl-pyrophosphate transferase. The crude extracts of *S. fragilis* contain apparently in addition another type of uridyl transferase which is independent of inorganic pyrophosphate (the rate is independent of the presence of pyrophosphate) even with the addition of pyrophosphatase to remove catalytical amounts of inorganic pyrophosphate. This uridyl transferase operates apparently as follows:



in which $\text{O}-\text{P}-\text{O}-\text{Gal}$ signifies α -galactose-1-phosphate. Since 6-phosphogluconic acid and reduced TPN are formed, if TPN is added the enzyme does not operate like a transglycosidase but like a uridyltransferase in which uridyl anhydride is transferred from α -glucose-1-phosphate to α -galactose-1-phosphate. This is a type of reaction suggested by Leloir as a preliminary step to his galacto-Waldenase reaction (188).

There are, therefore, three different ways of forming URPPGal: (a) uridylpyrophosphate transferase of "galactose adapted yeast," UTP and galactose-1-phosphate, (b) uridyl-galactosylphosphate transferase of "galactose adapted yeast" with URPPG and galactose-1-phosphate, and (c) galacto-Waldenase from "galactose adapted yeast" with URPPG alone. The last reaction will be discussed elsewhere.

There are indications that another uridyldiphosphoglycosyl compound in which the glycosyl part has been identified as N-acetyl glucosamine [Cabib *et al.* (44)] can react with pyrophosphate. It has been found that liver nuclei catalyze the formation of UTP in the presence of URPP-acet. gluc. amine [Smith *et al.* (288)]. A demonstration of the reverse reaction would be of great interest. Dutton & Storey (82) report that the cofactor of glucuronide synthesis contains uridylic acid and glucuronic acid. It has so far not been possible to demonstrate UTP formation from this cofactor nor from the

uridinediphosphoglycosyl compounds from penicillin-treated *Staphylococci* [Park (248)]. A number of related compounds isolated by Dowex chromatography from yeast (44), liver (132), and oviduct (297) will obviously be of much interest in connection with their possible biosynthesis from UTP and the corresponding glycosylphosphates.

BIOSYNTHESIS AND PROPERTIES OF URIDINETRIPHOSPHATE (UTP)

UTP can be formed by the following enzymic reactions: (a) uridyl-PP transferase plus URPPGlucose and PP (234), (b) "Nudiki" plus ATP and UDP (19), and (c) phosphopyruvate kinase, phosphopyruvate and UDP (167). From (a) and (b) UTP has been isolated through paper or column (Dowex-1) chromatography (234). UTP is much more acid-stable than ATP (234); the relation is like that between UDP and ADP (49a). UTP can donate its terminal P for the phosphorylation of ADP (19), but not for the phosphorylation of creatine or glucose. (20). UTP in the presence of the proper uridyl transferase can react with α -glucose-1-phosphate to give UDPG and with α -galactose-1-phosphate to give a mixture of UDPGal and UDPG. It has been suggested that UDP can operate as a carrier in glycosyl transfer (151), and the nature of the cofactor of glucuronide synthesis encourage at least a belief that URPP-glycosyl compounds can function as glycosyl donors. The "recharging" of UDP may be performed by glycosyl compounds like di- or polysaccharides or glycosyl phosphates. Another possibility which applies only to galactokinase, in which the sugar is phosphorylated in the 1-position, is a kind of kinase which forms a di-ester, i.e., ARPPPGal which undergoes fission between the third P and Gal by the terminal phosphate of uridine diphosphate. This phase may also require phosphorylation to UTP and then a subsequent transfer of "uridyl" to a glycosylphosphate. The uridyl transferase may form an intermediary "uridyl-enzyme."

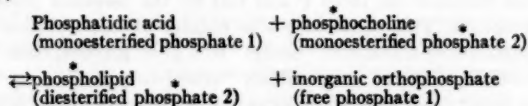
The most direct proof that UDPG can act as a glycosyl donor is by Leloir & Cabib (191), who show that UDPG and glucose-6-phosphate form UDP and trehalosephosphate. This will be discussed in detail in the section dealing with phosphorylases and transglycosidases. The uridyl glycosylphosphate transferase of *S. fragilis* catalyzes an exchange between free P³²-labelled α -glucose-1-phosphate and the glucose-1-phosphate in UDPG (151). Such an exchange is a requirement for the postulation of an intermediary "uridyl" enzyme but very far from proof. When further isolation of uridyl transferases has been obtained, direct proof for the formation of such a complex should be feasible.

HYDROXYL KINASES

General remarks.—This type of reaction is accompanied by large negative ΔF under most physiological conditions and will therefore proceed very far towards the side of phosphorylation of the hydroxyl group. This type of reaction may occur in two steps. The first is the formation of a condensation product which subsequently undergoes fission by water or by another hy-

droxyl compound. In a discussion dealing with active transport of glucose through membranes (i.e., intestinal epithelium) a suggestion was made by Rosenberg (269) that hexokinase might give rise primarily to the formation of glucosemetaphosphate (a nonelectrolyte considered suitable for passing through cell membranes) which subsequently would undergo a hydrolytic cleavage, the latter being practically irreversible. Since yeast hexokinase does not catalyze the uptake of O^{18} from water into any of the products of the reaction this type of fission apparently does not take place with this enzyme. One could also raise the objection that the reactions probably have a large positive ΔF and would therefore be ill suited to perform concentration work. However, if a sterol, for instance, would react with the anhydrous ester, forming a di-ester containing a hydrophobic group, such a compound might be more suitable for action transport through membranes [cf. Rosenberg (269)].

The reviewers stress some of these aspects mainly because aerobic phosphorylations with O^{18} enriched orthophosphate has revealed that the loss of O^{18} by dilution from water is of such proportions as to suggest intermediary formation of di-esters [Kalckar (151a); Cohn (62)]. The formation of a di-ester from ATP and phosphoglycerate [Baldwin (11)] has been disproved by Cohn (62). The formation of a di-ester through metaphosphoric ester in choline phosphorylation by choline kinase [Wittenberg & Kornberg (327)] is unlikely if phosphatidic acids as well as phosphocholine are intermediaries in phospholipid synthesis since it has been shown that the ratio between P^{32} and C^{14} in the latter remains unaltered in the phospholipids investigated [Kornberg & Pricer (169)]. This would suggest di-ester formation by the following pathway:



The di-ester formation in this case would scarcely go by the way of a metaphosphoric ester formed primarily through the action of a hydroxyl kinase. However, the hexokinases and related kinases of mitochondria and skeletal muscle have not as yet been subjected to inspection with the O^{18} isotope. Such an investigation seems worthwhile in view of the above mentioned paradox with respect to the dilution of O^{18} phosphate as well as for the clarification of problems like the action of hormones or the role of structural elements in metabolism.

HEXOKINASE

The phosphate donor is ATP. ITP and UTP may not act directly but only through ADP in the presence of "nudiki" [Berg & Joklik (19)].

The effect of the composition of the diet on the hexokinase activity in intestinal mucosa of rats has been investigated by Long (207). The results showed that the enzyme activity was higher when the rats were fed fat-free diet and lowest when they were fed a high-fat diet.

Hexokinase from brain has been found by Crane & Sols (69) to be associated with the particles. It was not possible to solubilize the enzyme activity by a number of different procedures. From acetone powder of the enzyme preparation 5 per cent of the activity could be extracted, but this activity sedimented at 100,000 g. Purification of the isolated particles was achieved by treatments first with lipase and then with deoxycholate, giving in total about 50-fold purification. The inhibition of brain hexokinase by G-6-P has been studied in detail. The relative activity of the brain enzyme towards different substrates has been studied by Sols & Crane (292), and the K_m values were determined. ADP is a competitive inhibitor while none of the phosphorylated reaction products, except G-6-P, inhibit the enzyme. It appears that the inhibition by hexose-6 phosphates depends on the configuration at carbon 2 while phosphorylation of free hexoses depends on configuration from carbon 3 to 6. The parasite *Schistosoma mansoni* is found by Bueding & MacKinnon (42) to possess high hexokinase activity [cf. also Eeg-Larsen & Laland (83)]. Some of the properties for instance with regard to inhibitors, resemble those of the brain enzyme. It differs from the brain enzyme with respect to specificity and optimum concentration of ATP and Mg^{++} . Liebecq (200) found the optimal relative molar concentrations of Mg^{++} and ATP to be unity for hexokinase from muscle extract, the pH optimum of which was 7.8 to 7.9. From the studies of Saltman (275) it seems that the distribution of hexokinase activity between the soluble and insoluble part of extracts of higher plants to a larger extent is dependent on the extraction procedure. This finding, in connection with the similarity of the properties of the enzyme in the two fractions, suggests that all of the enzyme is present in insoluble form *in vivo*. The properties of the enzyme from wheat germ with regard to specificity, Michaelis constants, and optimum conditions were investigated. Among a number of inhibitors of the rather unfractionated enzyme were DNP,² alloxan, and heavy metals. Evidence for hexokinase activity in white blood cells has been obtained by Wagner & Yourke (314). The enzyme activity is associated with the insoluble particles. Specificity studies by Eeg-Larsen & Laland (83) revealed that of a large number of carbohydrates and carbohydrate derivatives only D-glucose, 2-deoxy-D-glucose, D-mannose, and D-fructose served as substrates for hexokinase from yeast. Evidence for 2-deoxy-D-glucose being phosphorylated by ATP in the presence of yeast hexokinase has been obtained also by Cramer & Woodward (67). 2-Deoxy-D-glucose furthermore seems to inhibit the phosphorylation of glucose by the hexokinase system. Hexokinase and galactokinase have been found in extracts from *Lactobacillus bulgaricus* by Rutter & Hansen (270). There is indication that UDPG may play a role in promoting synthesis of galactose-metabolizing enzymes other than galactokinase. De Ley (73) and Vermeulen & De Ley (312) describe a group of enzymes observed in crude extracts of *Aerobacter cloacae* which can phosphorylate hexonic acids, which they term hexonokinases. Hexokinase is a constitutive enzyme. Kinases for D-galactonate, D-gluconate, 2-ketogluconate, D-ribose, and D-xylose, were all formed as

adaptive enzymes. Novelities are D-galactonate kinase and 2-ketogluconate kinase.

Phosphorylation of pentoses.—A cell-free extract of *E. coli* adapted to ribose was found by Heald & Long (113) to utilize ribose in the presence of ATP and Mg^{++} . The phosphorylated product was isolated and all of its properties suggested it to be ribose-5-phosphate.

Lampen (184) found that extracts of *Lactobacillus pentosus* grown on pentoses contain an enzyme which catalyzes the phosphorylation of xylulose. The product consisted mainly of a mixture of ribose-5-phosphate and ribulose-5-phosphate. The experiments gave no information about the primary product of phosphorylation or at which state the xylose configuration is converted to the ribose configuration.

Triosekinase.—Evidence for a triosekinase from guinea pig has been obtained by Hers & Kusaka (118). The enzyme is reported to phosphorylate D- and L-glyceraldehyde at the same rate and dihydroxyacetone at half the rate.

Phosphofructokinase.—Muntz (236) reported that a fraction from brain extract phosphorylates fructose-6-phosphate in the presence of either ATP or ITP. The activity is lost after the enzyme has been passed through a sodium-charged cation exchange resin. The activity is restored by addition of certain bivalent ions plus ammonia. K^+ activates the crude but not the purified enzyme. The affinity of the enzyme for Mg^{++} , Mn^{++} , Ca^{++} , and K^+ was determined. Phosphofructokinase activity has been found in a great variety of plants [Stumpf (298)]. The optimum conditions for the enzyme in water extracts of pea meal have been studied. Studying the phosphorylation of fructose by ATP and the enzyme from muscle, Hers (117) found that in the presence of very high concentrations of fructose the product is fructose-1-phosphate. Under these conditions the 1-phosphofructokinase activity is low, compared with that of fructokinase and 6-phosphofructokinase. Sorbose was phosphorylated by the same enzyme. Hers (116) has also studied an enzyme from beef liver which phosphorylates fructose and sorbose but not aldoses. Fructose is phosphorylated in the 1-position. The reaction requires Mg^{++} and is activated by K^+ . In the presence of low K^+ concentrations the optimum Mg/ATP ratio is 0.5 and in the presence of high K^+ concentration (1M) this ratio becomes 1. The active substrate appears to be an ATP-Mg complex. The affinity of the complex for the enzyme is increased by the presence of K^+ . In the presence of very high concentrations of K^+ this ion competes with the ATP-Mg complex for the enzyme.

Phosphorylation of galactosamine.—Cardini & Leloir (50) have found that a purified liver fraction catalyzed the phosphorylation by ATP of galactose and galactosamine with the same rate. Galactosamine phosphorylation was very markedly inhibited by the presence of galactose, and galactose phosphorylation was inhibited to a rather small extent by the presence of galactosamine. Extracts of *S. fragilis* also catalyzed the phosphorylation of galactosamine, and this enzyme activity increased like the galactokinase activity,

when the organism was grown on galactose instead of glucose. The reaction product was galactosamine-1-phosphate. The authors suggested that the enzyme responsible for this reaction is galactokinase.

Phosphoribokinase.—The existence of a kinase phosphorylating ribose phosphate has been inferred by the work of Williams & Buchanan (326) and Saffran & Scarano (273). Both groups have found that ATP in addition to ribose-5-phosphate and a purine (hypoxanthine or adenine) greatly stimulates the rate of incorporation of the purine into a 5-nucleotide (5-inosinic acid, 5-adenylic acid). Scarano (277) has purified an enzyme from pigeon liver which catalyzes the phosphorylation of ribose-5-phosphate, presumably in the 1-position since the ester formed is active in promoting incorporation of adenine in the absence of ATP. Ribose-1-phosphate was not active as phosphate acceptor in the purified system. Since the preparation contains phosphofructokinase and it has not as yet been tested for ribose-5-phosphate isomerase [Axelrod & Jang (10)], the possibility that ribulose-5-phosphate is being phosphorylated in the 1-position cannot at present time be ruled out.

N-Riboside kinases.—To this class of kinases typified by the phosphorylation of adenosine and 2-aminoadenosine to the corresponding 5-nucleotides [Ostern & Terszakowec (246); Caputto (49); Kornberg & Pricer (168)], has been added an interesting newcomer. Greenberg (109) has recently found that the 5-amino-4-imidazole carboxyamino riboside can be phosphorylated by dialyzed pigeon liver extract or an autolysate of dry brewer's yeast in the presence of ATP, phosphoglyceric acid, and Mg^{++} . The product was isolated by ion exchange chromatography and found to be carboxamine-5'-phosphoriboside. This reaction may be important not only for the formation of inosine-5-monophosphate (cf. also 41) but perhaps also for the formation of the corresponding diphosphate. It would therefore be of interest to know whether Greenberg's system can form the diphosphate. So far it has not been possible to find a kinase for inosine or IMP making IDP. The latter might be formed by deamination from ADP [Webster (322)].

Choline kinase.—Wittenberg & Kornberg (327) have purified an enzyme, cholinephosphokinase (or more consistent with the previous terminology cholinekinase), from dry brewer's yeast. By fractionation including ammonium sulfate precipitation, and calcium phosphate gel adsorption a 25-fold purification was obtained. The phosphorylated product was isolated and identified as phosphocholine, which was shown to be the main product of phosphorylation. The enzyme reaction had a pH-optimum between 8.0 and 9.5, required Mg^{++} , and was activated by cysteine. Other substrates for the enzyme were dimethyl- and diethylaminoethyl alcohol, monoethyl- and monoethylaminoethyl alcohol, and aminoethyl alcohol. The affinity of the enzyme for these compounds, however, was much lower than that for choline and ATP. The enzyme was present in a number of tissues from different animals. A similar enzyme is probably present in the system for acetylcholine synthesis by Berry & Stotz (24) using a brain preparation.

Phosphorylation of DPN and dephosphorylated CoA.—Wang (318) has sep-

arated from pigeon liver the DPN phosphorylating enzyme from the enzyme which phosphorylates dephosphorylated CoA. The two enzymes were partly purified. TPN can be formed quantitatively with the DPN kinase system whereas reduced DPN is not phosphorylated.

Phosphorylation of thiamine.—Leuthardt & Nielsen (196) have purified from the soluble part of rat liver extract an enzyme which catalyzes the formation of thiaminepyrophosphate from thiamine and ATP. The enzyme reaction required Mg^{++} and a large excess of ATP over thiamine, the pH-optimum was at 6.8 to 6.9. Both AMP and ADP inhibited the reaction. The reaction was considered to be a transfer of a pyrophosphoryl group of ATP, since the formation of cocarboxylases was much slower when thiaminemonophosphate was used as substrate instead of thiamine itself. With a purified thiamine-pyrophosphorylating enzyme, experiments with ATP labelled with P^{32} in the terminal phosphate group might be valuable. Provided that no adenylate kinase is present a definite proof might be obtained for the transfer of the pyrophosphate group. In that case the sequence of P^{31} and P^{32} in the thiamine pyrophosphate formed would add further information on the mechanism of the reaction. Thiamine triphosphate has recently been found in yeast (158).

Kinases involved in the biosynthesis of CoA.—Novelli (243) has found that pantetheine and ATP in the presence of a specific kinase bring about formation of phosphopantetheine, the phosphate group being esterified to the hydroxyl group of the pantoic acid. The phosphorylated product reacts apparently once more with ATP, this time becoming adenylated (see section on nucleotidyl transferase) forming the so-called phospho-CoA.

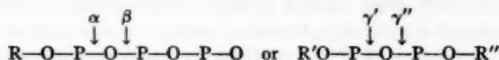
Another kinase catalyzed the phosphorylation in the 3-position of the ribose to CoA [phosphorylation of DPN to TPN (317, 318)]. Hurwitz (134) has purified a kinase from yeast which catalyzes the phosphorylation of pyridoxal, pyridoxamine, pyridoxine, deoxypyridoxine, and 4-5 diphydroxy-2-methylpyridine.

ADENOSINETRIPHOSPHATASES, APYRASES, AND ORGANIC PYROPHOSPHATASES

General problems.—With most of these enzymes, there is the possibility of coupled phosphorylated (or pyrophosphorylated or adenylated) intermediary complexes with groups in the enzyme protein. For this reason isotope-exchange studies and especially application of the O^{18} isotope to establish the place of the fission are of paramount interest. Thus, Koshland and his co-workers have found that not only regular but also myosin adenosinetriphosphatase catalyzes a split between oxygen and the terminal P [Clarke & Koshland (59, 173)]. Moreover, the nucleotide part seems to be of less importance with respect to specificity since UTP is hydrolyzed somewhat faster than ATP by "heavy" meromyosin [Geller *et al.* (97)]. If myosin adenosinetriphosphatase should be of importance for an "ATP-chemo-mechanical" geared effect on the contractile mechanism [Engelhardt *et al.* (86); Szent-Györgyi

(302); Weber (320, 321; Hill (122)), it might perform the coupling through phosphorylation of myosin [Kalckar (147); Riseman & Kirkwood (264)]. If a chemo-mechanical coupling under certain circumstances can be reversed into a mechano-chemical coupling [cf. Hill (121); Pryor (255)], it should perhaps be possible to obtain a reversible exchange like that described under glutathione synthesis [Snoke (291)]. The problem of ATP, meromyosin adenosinetriphosphatase, and contraction is, of course, highly complex since ATP concentration and especially the role of Ca^{++} and K^{+} are factors of such primary importance. A "phosphoryl transferase" might be detected in magnesium actomyosin preparation and studied with advantage in a Weber-Engelhardt actomyosin fiber. Inhibition studies using calcium and magnesium suggest that dephosphorylation of ATP is not directly involved in the shortening of myosin thread (31).

Concerning the biosynthetic potentialities of enzymes classified as organic pyrophosphatases catalyzing the following types of reactions:



we know already that the liberation of inorganic pyrophosphate from pure ATP (arrow α and β) can be coupled with biosynthetic reactions presumably through formation of nucleotidyl-enzyme (arrow α) or pyrophosphoryl enzyme (arrow β). The splitting of pyrophosphate dinucleotides (type γ' or γ'') although seemingly a simple hydrolysis could involve steps like that observed in ammoniacal hydrolysis of certain pyrophosphate dinucleotides, i.e., formation of cyclic pyrophosphate esters. Thus, the splitting of a new flavin dinucleotide [Huennekens *et al.* (131)] by nucleotide pyrophosphatase reported to yield cyclic riboflavin 4,5'-phosphate might be interpreted that nucleotide pyrophosphatase catalyzes the primary formation of such a compound.

ADENOSINETRIPHOSPHATASES

Insoluble adenosinetriphosphatases.—Kielley & Kielley (157) found that the adenosinetriphosphatase activity of liver mitochondria was more than doubled when these were broken. This phenomenon was also encountered by Sacktor (272). They found that an insoluble part (sedimented at 110,000 g) of the broken particles splits off only one P_i from ATP, whereas intact mitochondria liberate two P_i from ATP. This insoluble part had similar but lower activity towards ITP and little or no activity towards AMP, P-P, IDP, ADP, and thiamine pyrophosphate. The optimum conditions for the enzyme with regard to pH and Mg^{++} concentration were determined. ADP and high magnesium ion concentrations were inhibitory. In view of the possibility of a formation of phosphoryl-X-enzyme in some of these enzyme systems ADP inhibition is of considerable interest. The adenosinetriphosphatase and inosinetriphosphatase activity of a suspension of mouse spleen homogenates was found by Ashwell & Hickman (8, 9) in contrast to the enzyme of Kielley &

Kielley to be activated not only by Mg^{++} but also by Ca^{++} . The enzyme was unchanged under quite variable conditions. After total body x-irradiation the properties, the total amount of the enzyme, and its distribution in the tissue fractions (220) in spleen was unchanged. As a result of a 70 per cent weight loss of the spleen the specific activity, however, was markedly increased. This increase was ascribed to radioresistance of the adenosinetriphosphatase. Under such conditions a similar but less pronounced increase in specific activity was observed for the 5-nucleotidase and the alkaline phosphatase from spleen. Swanson (300) found that the adenosinetriphosphatase activity of rat liver mitochondria has two distinct pH optima, the location of which depended on the concentration of Mg^{++} and the nature of the buffer. Thyroxin and triiodothyronine were found by Feldott (87) to stimulate the adenosinetriphosphatase of pre-aged but not of fresh mitochondria and certain steroids exert inhibitory actions on myo-adenosinetriphosphatases (230). Lardy & Wellman (185) found that DNP greatly enhances the adenosinetriphosphatase activity of the nuclear and the mitochondrial fractions of liver. The effect changed appreciably, however, under variable conditions depending on salt concentrations of the medium, pre-ageing of the mitochondria, and presence of Mg^{++} and Ca^{++} . The authors pose the interesting problem whether a DNP-enzyme (covalent linkage) may be formed and rapidly hydrolyzed. Some dicarboxylic acids and *p*-chloromercuribenzoate depressed the effect of DPN whereas the dicarboxylic acids themselves, like fatty acids, enhanced the phosphate liberation from ATP. DNP shows a similar effect with myomitochondria (53).

The numerous observations that oxygen consumption in mitochondria depends on the degradation of ATP either by transphosphorylation or by dephosphorylation [Potter *et al.* (254); Niemeyer & Jolil (241); cf. also Slater's review (286)] are noteworthy. The compulsory coupling between phosphorylation of phosphate acceptors and the oxidation reduction of the fermentations was first demonstrated by Meyerhof and his group (225) and largely clarified by Warburg and co-workers. Similarly Witter *et al.* (328) found that DNP activates the hydrolysis of ATP and also of inorganic pyrophosphate by washed mitochondria, under anaerobic conditions and in the absence of substrates for oxidation. This is not the case, however, if the mitochondria have been prepared in hypertonic sucrose-isotonic KCl solutions. From studies on the oxidation of malate they concluded that the uncoupling effect of DNP in this case is not attributable to the activating effect of the adenosinetriphosphatase activity. DNP was found by Dresel (80) to cause the accumulation of hypoxanthine and uric acid in the bird liver preparation as a result of enhancement of the breakdown of the adenine nucleotides. The nucleic acid breakdown was uninfluenced by DNP. Kitiyakara & Harman (159) found adenosinetriphosphatase activity of pigeon breast muscles to be associated with mitochondria and with the myofibrillar fraction.

Soluble adenosinetriphosphatases.—Gilmour & Calaby (101) found that almost all of the Ca -activated adenosinetriphosphatase of insect muscles

(100) is associated with the actomyosin fraction and almost all of the Mg-activated adenosinetriphosphatase is present as a water-soluble enzyme. The myosin-adenosinetriphosphatase is predominant in the less active femoral muscle whereas in the flight muscle the soluble adenosinetriphosphatase is responsible for 50 to 70 per cent of the total adenosinetriphosphatase activity. They purified the water-soluble enzyme and separated it from inorganic pyrophosphatase. Since the adenylate kinase activity of the preparation was negligible and since two molecules of orthophosphate were released from ATP or ITP, it was concluded that ADP and IDP are also substrates. ADP was a competitive inhibitor for the ATP breakdown. The enzyme-substrate dissociation constants for ATP and ADP were found to be 8.6×10^{-4} M and 1.6×10^{-3} M, respectively. The authors related the high Q_{10} (greater than 4) of this adenosinetriphosphatase to the dependance on high temperature for muscular activity in insects.

Three different adenosinetriphosphatase activities have been separated from bull seminal plasma by Heppel & Hilmoe (114). From aged plasma an "acid" adenosinetriphosphatase was fractionated free from the P-P forming enzyme. This enzyme was activated by Mg^{++} , to a lesser extent by Mn^{++} and inhibited by Ca^{++} . ADP is split relatively slowly by this enzyme. The activity of a partly purified alkaline adenosinetriphosphatase was approximately doubled by the addition of Mg^{++} or Ca^{++} . ADP was split at almost the same rate as ATP. An enzyme forming one P-P from each ATP [cf. also Siliprandi & Cerletti (282)] was purified 34-fold. $CaCl_2$ and $MgCl_2$ have no effect on this enzyme; it has pH optimum at 8.4 to 9.0 and is fairly heat-stable. P_i is released from ADP by probably the same enzyme. Unfractionated seminal plasma was stimated to contain 80 units of acid adenosinetriphosphatase, 130 units of alkaline adenosinetriphosphatase, 30 units of P-P forming adenosinetriphosphatase, 30 units of inorganic pyrophosphatase, and 2900 units of 5-nucleotidase. Snake venom and clear extracts of rabbit kidney also form P-P from ATP and (in the case of snake venom) from ITP. Similar results were obtained by Johnson *et al.* (140) using unfractionated cobra venom. A pyrophosphate-forming enzyme with pH optimum at 8.4 was found; ADP was slowly converted to P_i and adenosine by the enzyme. Experiments with labelled ATP showed that the P-P was derived from the two terminal phosphate groups of ATP. Antiserum was found to be a potent inhibitor of the 5-nucleotidase and also, to smaller extent of the hydrolysis of ATP and ADP. The P-P forming enzyme and the adenosinetriphosphatase were suggested as being components of the toxic principle of the venom.

Hermann *et al.* (115) have found one enzyme in liver (a) which catalyzes the dephosphorylation of ITP to IDP, and another (b) which catalyzes the splitting of ITP to inosinic acid and inorganic pyrophosphate. They also found an organic pyrophosphatase which was less temperature-stable than the two former. By fractionation on tricalcium phosphate gel they separated the two enzymes.

The hydrolysis by nucleotide-pyrophosphatase of a new flavin dinucleotide

[Huennekens *et al.* (131)] was reported to yield cyclic riboflavin-4,5-phosphate identical with that described by Forrest & Todd (93). The interesting finding might mean that certain nucleotide pyrophosphatases form cyclic esters as intermediary compounds and, depending on the substrate, the cyclic esters accumulate to a detectable extent.

Adenosinetriphosphatase activity of myosin.—Laidler & Ethier (182) have tried to determine heats of entropies associated with the formation of a myosin adenosinetriphosphatase-ATP complex. Mixed solvents, which allow entropy terms to be resolved into electrostatic and nonelectrostatic (structure) contributors were used. The rate of adenosinetriphosphatase activity increased as the concentration of methanol and dioxane increased (optimum concentrations for methanol 30 per cent and for dioxane 20 per cent). Half of the entropy increase was accounted for as an electrostatic effect, presumably a neutralization of positive charges of the protein by ATP. Addition of ATP brings about a change of particle shape of myosin [Morales & Botts (231)]. Kafani & Engelhardt (146) have recently shown that ATP can bring about an anisodimensional contraction of myosin fibers which seems completely free of actin as judged by the absence of the viscosity changes. In contrast to actomyosin which shows its pH optimum for contractility at 7 the myosin ATP contraction reaches its maximum at pH 9. The authors observe that the pH optimum of adenosinetriphosphatase activity is shifted likewise [Sarkar *et al.* (274)], which once more poses the question whether the effect of ATP in the myosin or the actomyosin structure is dependent on adenosinetriphosphatase activity. Blum & Morales (28) have investigated the reaction between ATP and solutions of myosin. Using light scattering measurements they find that the molecular weight of myosin, though different for different preparations, remains fairly constant during the interaction between myosin and ATP, which indicates that ATP does not depolymerize the myosin. The distribution of adenosinetriphosphatase activity between myofibrillar proteins and the sarcoplasmic fraction in the developing chick embryo has been studied by Robinson (266). In the earliest embryos the sarcoplasmic enzyme activity was very high compared with that of the myofibrillar fraction. As the development proceeds, however, this proportion gradually changed until almost all of the activity was present in the myofibrillar portion. The properties of the sarcoplasmic enzyme resembled more those of the myosin enzyme activity than those of the water extractable enzyme of Kielley & Meyerhof. The effect of trypsin digest on myosin has been studied by Gergely (98). Whereas some of the typical properties of myosin were changed by such treatments and viscosity measurements indicated profound changes in molecular structure, the adenosinetriphosphatase activity was unchanged. The specific adenosinetriphosphatase activity of trypsin digested myosin was increased about 2.5-fold by fractionation. The trypsin-treated myosin has a higher Michaelis constant for ITP than for ATP. ITP which is itself a substrate for myosin inhibits the myosin-adenosinetriphosphatase activity. Mihalyi & Szent-Györgyi (228) divided the tryptic digest of myosin into two

time phases. In the first phase the adenosinetriphosphatase activity was unaffected whereas some of the other properties of myosin were changed. After longer digestion, however, the enzyme activity decreased markedly. During the first phase two components were obtained by ultracentrifugation. The faster sedimenting component "heavy meromyosin" has a specific activity higher than the whole digest, and the slow sedimenting component has very small specific activity. The specific activity of myosin therefore seems to be linked to a subunit, the activity of which is independent of whether it is free or built into the native myosin molecule.

UTP is hydrolyzed to UDP and P by this preparation, and preliminary studies [Geller *et al.* (97)] show that the rate is somewhat higher than the corresponding hydrolysis of ATP.

Munch-Petersen (235) has studied the dephosphorylation of ATP during the contraction of striated muscles. The content of ATP, ADP, and AMP of muscles after rapid freezing and extraction with perchloric acid was determined by ion exchange chromatography. Tortoise muscles fixed in the initial phase of twitch showed a significantly higher ADP content and a correspondingly lower ATP content than that of the nonstimulated control muscle. It was further found that the contraction caused by rapid freezing of the muscles did not give rise to significant changes in the ADP content as compared to muscles extracted directly with perchloric acid. Tetanic contraction of curarized frog muscle did not give significant changes in the ratio between the ATP and the ADP content. If the muscles, however, are fatigued with a number of single contractions the main part of ATP is broken down to IMP.

The mechanism of the adenosinetriphosphatase activity of lobster muscle strips have been studied with H_2O^{18} containing water by Koshland & Clarke (173). The data show that the reaction involved a splitting between O and the terminal P by nucleophilic displacement of the terminal phosphorus atom. The O^{18} content of the isolated P_i was, however, higher than would be expected from a simple hydrolysis. From the metabolic rate of the lobster muscle preparation the authors concluded that this increased O^{18} content is caused only to a minor part by recycling, and is attributable to a transfer of oxygen between orthophosphate and H_2O catalyzed by the muscle strips. [cf. also (59)]. The presence of glycogen, phosphorylase, and a hexose phosphatase in the preparations might also contribute. The authors have these aspects under investigation. Novikoff *et al.* (244) found no essential differences between the intracellular fractions of adenosinetriphosphatase activity of regenerating and of normal rat liver.

Adenosinetriphosphatase in red cells.—Straub *et al.* (96, 301) have studied the adenosinetriphosphatase of the erythrocytes from various mammals as related to the mode of hemolysis. The intact membranes of erythrocytes contain an adenosinetriphosphatase which apparently catalyzes the liberation of all three phosphates of ATP as inorganic orthophosphates. Besides this enzyme is an inorganic pyrophosphatase. The adenosinetriphosphatase is supposedly located on the inside of the membrane, but is apparently in an

inactive form in the nonhemolyzed cells. The enzyme requires Mg^{++} for its activity, is inhibited by fluoride, and has a broad pH optimum between 7 and 8. If the rate of splitting of ATP is called 100 per cent, the rates of splitting of ADP and 5-adenylic acid are about 67 per cent. The α - and β -glycerolphosphate of phenylphosphate are hydrolyzed very slowly. The activation of adenosinetriphosphatase is brought about by freezing, whereas osmotic hemolysis does not alter the membrane of the human red cell in such a way as to give rise to adenosinetriphosphatase activity. Hemolysis by organic solvents and many other ways of moderate damage to the membrane do not bring about any adenosinetriphosphatase activity, presumably because of denaturation of this enzyme, although the activities of a number of synthesizing enzymes were more or less intact. Adenosinetriphosphatase activity has been observed in white blood cells by Wagner & Yourke (314). The adenosinetriphosphatase of cartilage has also been subject to study (252).

GLYCOSYL-PHOSPHATE TRANSFERASES- (PHOSPHORYLASES)

In accordance with the program outlined in the introduction, the name glycosyl-phosphate transferase should indicate (a) that the anhydrous moiety transferred is a glycosyl group and (b) that phosphate can act as an acceptor of the glycosyl group, forming an α or a β glycosyl phosphate. For discussion of configurational aspects, especially inversion of glycosyl linkages in connection with the fission of the C—O bond, the reader is referred to three review articles: Hestrin (119), Kalckar (152), and Koshland (171, 172). A newly discovered biosynthetic reaction for sucrose synthesis [glycosyl-UDP transferase (191)] will be described later in this section.

Phosphorolysis of maltose.—An enzyme from *Neisseria meningococcus*, maltose phosphorylase, catalyzes the reaction: Maltose (1-6 glycosidic linkage) \rightleftharpoons β -glucose-1-phosphate + glucose [Fitting & Doudoroff (90)]. Quite apart from the fact that the enzyme catalyzes the formation of a β -ester from an α -glucoside, this reaction differs in many other important points from that catalyzed by sucrose phosphorylase. The sucrose phosphorylase catalyzes a rapid exchange between inorganic phosphate and the α ,1-ester phosphate (77) whereas the maltose phosphorylase catalyzes the corresponding exchange with the β -ester exclusively in the presence of the other partner, i.e., glucose. Moreover, arsenolysis of the β -ester requires the presence of glucose. Fitting & Doudoroff assume that the maltose phosphorylase requires the following situation in order to operate specifically: α -glucosido-glucose-enzyme-phosphate or β -glucosido-phosphate-enzyme-glucose. These two complexes may be the active intermediate. This type of reaction may correspond to what Koshland (172) calls "single displacement," and if this kind of reaction occurs at an asymmetric carbon, it generally leads to inversion. The sucrose phosphorylase on the other hand should belong to the type of "double displacements" and thus be able to retain the configuration ("retention"). The possibility should be tested that the phosphorylases

which do not catalyze an enzymic exchange between free phosphate and the glycosyl-bound phosphate, might catalyze an exchange between the free and the phosphorylated sugar (a 3-step exchange, see section on nucleotidyl transferase). In the case of polysaccharide phosphorylase it has been tested, and no exchange with C^{14} -labelled glucose was found. [Cohn & Cori (63)].

Campbell *et al.* (46) investigated the inhibitory action of a number of monosaccharides and glucosides on the synthesis of polysaccharide from α -glucose-1-phosphate, using crystalline muscle phosphorylase. In addition to D-glucose only α -methylglucoside and α -phenylglucoside inhibited the reaction. This finding was taken as further confirmation of the α , β specificity of phosphorylase. Nakamura (239) has, in contrast to previous investigators, found that lima bean phosphorylase requires soluble starch as a primer for the formation of polysaccharide from G-1-P. The enzyme primer dissociation constant was determined to be 300 mg. of Lintner's soluble starch per liter.

The interesting enzyme conversion of muscle phosphorylase *a* into *b* has been studied further by Keller & Cori (155). Phosphorylase *b* has a molecular weight one-half that of *a* and the enzyme performing this process was termed the "phosphorylase rupturing enzyme." It apparently converts one molecule of *a* into two molecules of *b*.

A simple procedure for obtaining crystalline phosphorylase from potato juice has been described by Baum & Gilbert (13), based on the observation that the presence of amylase in potato juice results in a strong and selective precipitation of the enzyme at an ethanol concentration at which the enzyme is otherwise soluble. The combination of this procedure and ammonium sulfate fractionation results in the crystalline enzyme in a few steps. Crystalline Q enzyme can be obtained as a by-product. The effect of epinephrine hypoglycemic factor and other compounds on phosphorylase has recently been summarized by Sutherland (299).

Nucleoside phosphorylase.—Kritskii & Melik-Sarkisian (178) report that nucleoside phosphorylase from mammalian tissue also shows trans-N-glycosidic activity, bypassing a phosphorylated stage. They also found a trans-N-glycosidase which is devoid of any phosphorolytic activity (cf. also 135). This is the first report of a trans-N-glycosidase in the riboside series.

In a discussion concerning the general importance of the formation of glycosyl-enzymes it is of essential interest to state that the nucleoside phosphorylases of ribosides or deoxyribosides show no exchange between inorganic phosphate and the 1-ester phosphate unless the other partner is also present (94). Thus, what is considered the first prerequisite for the formation of a covalent linkage of an enzyme group with the anhydrous moiety of the substrate is missing in these cases.

This enzyme has been extensively purified from beef liver acetone powder by Buchanan & Korn (40, 164). The procedure involves fractionation with ethanol and ammonium sulfate and adsorption on silica gel. Korn *et al.* (165) found that this enzyme preparation catalyzes the formation of 4-amino-5-

imidazolecarboxymide riboside from R-1-P (but not from R-5-P or free ribose) and the free base. The riboside was isolated by paper chromatography (propanol-water, R_F : 0.39, free carboxamide: 0.54) and analyzed. The ratio determined by ultraviolet absorption at the maximum 267 $m\mu$ of diazotizable amine to pentose (orcinol) was found to be unity. It was also found that the enzyme preparation which is free of adenase but contained an adenosine deaminase catalyzed the formation of inosine from adenine and R-1-P. This could be attributable to either a direct interaction or to an indirect one combined with a transglycosidation [cf. Kritskii & Melik-Sarkisian (178)] since most adenine preparations contain traces of hypoxanthine. Paegle & Schlenk (247) have isolated a specific uracil riboside phosphorylase from *E. coli*. The enzyme has a pH optimum at 7.2. Requirement for inorganic phosphate was demonstrated and R-1-P was isolated as a reaction product. An equilibrium mixture contained 62 per cent of uridine and inorganic phosphate and 0.38 per cent of uracil and R-1-P, respectively. The observation that 8-azoguanine is a substrate of nucleoside phosphorylase (93a) has gained further interest by the demonstration of azoguanine in the nucleic acid of plant viruses (219).

The distribution of purine nucleoside phosphorylase in the brain tissue of the monkey has been studied by histochemical technique [Robins *et al.* (265)]. The authors carefully controlled factors affecting kinetics such as substrate concentration, temperature, pH, time. There was no uricase present in the tissue preparation nor hydrolytic fission of inosine since the amount of hypoxanthine liberated corresponded mole for mole to phosphate incorporated. Based on inosine phosphorolysis per unit weight protein per hour they found high activity in the granular layer of cerebellar cortex, and the subadjacent white matter was particularly rich in the enzyme, whereas the molecular layer had markedly less activity. The inosine phosphorylase of the cerebellum seems therefore to be more abundant in a tissue poor in nuclei than in those rich in nuclei.

It is interesting here to recall the findings of Allfrey *et al.* (6), in which nucleoside phosphorylase was described as being relatively more abundant in the nuclei from liver, heart, and kidney. It is not quite certain, however, in the latter case whether nucleoside transglycosidases or hydrolases could have been included in the nucleoside phosphorylase value.

Nucleotide synthesis by alternative pathways than phosphorylation of nucleosides.—The existence of enzyme catalyzing the transfer of hydroxylphosphorylated "pentosyls" from nitrogenous bases to phosphate or in the opposite direction is indicated by work on the incorporation of labelled purine of purine precursors into nucleosides or nucleotides. Enzymatic studies by Wajzer (315) encouraged the belief that nucleotides could be formed by a pathway independent of nucleosides.

The work of Greenberg (109) using labelled formate as indicator pointed clearly to the possibility of a bypassing of the nucleoside stage in the incorporation of purine precursors into nucleotides.

This was furthermore encouraged by the finding [Williams & Buchanan (325)] that ribose-5-phosphate and ATP greatly stimulated the formation of inosinic acid from hypoxanthine. Recently, Williams & Buchanan (326) have shown that the conversion of hypoxanthine to inosinic acid by pigeon liver extracts is mediated by at least two enzymes which may be separated by ethanol fractionation: (a) an active fraction is precipitated below 15 per cent; (b) the other active fraction is precipitated between 20 and 45 per cent ethanol. Both the crude and the purified preparations require ribose-5-phosphate (a nucleoside) and ATP (4 to 5-fold enhancement of the conversion of hypoxanthine to inosinic acid by either one).

The formation of 5-adenylic acid in homogenates of pigeon liver from radioactive adenine [Goldwasser (104)] has been studied by Saffran & Scarano with respect to mechanism (273). Adenine C^{14} labelled in the 8 position (57) was used. Saffran & Scarano (273) also showed the existence of two enzyme systems the first of which requires ATP and ribose-5-phosphate (or the 1-ester) and can form a product which is used for the incorporation of adenine catalyzed by the second-step enzyme which is heat stable. When the kinase system was incubated prior to the adenine-incorporating system in the presence of ATP, it could be shown that the rate of 5-adenylate synthesis was enhanced about 100-fold by addition of either ribose-5- or 1-phosphate. It has been mentioned that the first step enzyme has been shown to phosphorylate ribose-5-phosphate [the corresponding 1-ester is not an active acceptor after some purification steps (277)]. The second step enzyme may be classified as a nucleotide phosphorylase, i.e., $\text{adenine} + \text{ribose-1,5-diphosphate} \rightleftharpoons \text{5-adenylate} + \text{phosphate}$. The participation of ribose-1,5-diphosphate in the adenine incorporation was further indicated by the fact that phosphoglucomutase incubated with ribose-1-phosphate and glucose-1,6-diphosphate brought about a 50 to 100-fold increase in 5-adenylate synthesis. The incubation of the three components generates ribose-1,5-diphosphate (161, 162). Since the reaction has not as yet been demonstrated in the reverse direction, it may be advisable to reserve judgment.

Transglycosidases.—An interesting new type of transglycosidation was found by Leloir & Cabib (191). UDPG can act as a glucosyl donor and the reducing group of glucose-6-phosphate as a glucosyl acceptor according to the formulation: $\text{UDPG} + \text{glucose-6-phosphate} \rightleftharpoons \text{UDP} + \text{trehalosemonophosphate}$. It is not as yet known whether the reaction proceeds to an appreciable extent in reverse. The reaction is catalyzed by an enzyme present in crude dialyzed yeast juice as well as in certain fractions obtained by precipitation with ammonium sulfate. The authors showed that for each mole of UDP formed one mole of acid labile glucose was consumed and one equivalent of reducing power disappeared. Paper chromatography revealed a slow moving phosphoric ester. Action of phosphatase on this material liberated a nonreducing sugar, having the same R_F value as trehalose ($\alpha\alpha'$ 1,1' glucosylglucosyl).

Buchanan (39) has recently found sucrose phosphate as an intermediary

in sucrose formation in sugar beets and has suggested that sucrose-phosphate is formed from UDPG and fructose phosphate.

Leloir & Cardini (194) most recently isolated an enzyme from wheat germ which can catalyze the following reversible reaction: $\text{UDPG} + \text{fructose} \rightleftharpoons \text{UDP} + \text{sucrose}$. It is of great interest that this reaction brings about synthesis of free sucrose; sucrose phosphate was not formed. The reaction is reversible, but the equilibrium greatly favors the synthesis of sucrose. The enzyme was obtained by extraction of wheat germ with phosphate pH 7, dialyzed and precipitated with ammonium sulfate. It was then three times precipitated at pH 5 and redissolved. The same type of enzyme could be obtained from corn germ and bean and potato sprouts. This type of enzyme which might be classified as a glycosyl-UDP transferase, is unable to catalyze the synthesis of sucrose from α -glucose-1-phosphate or α -glucose-1,6-diphosphate. The recent report by Turner (306) in which a plant enzyme was reported to bring about sucrose formation from glucose-1-phosphate in high yields is of interest in this connection. Perhaps Leloir & Cardini's system (194) is at work here too.

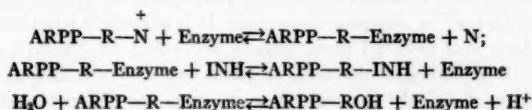
Rutter & Hansen (270) find that addition of UDPG to yeast grown in glucose greatly increases the rate of acid production from galactose. Since added α -galactose-1-phosphate (174) is utilized better than α -glucose-1-phosphate and since galactokinase is present in nongalactose adapted cells, the action of UDPG is still obscure. The interesting findings indicate that UDPG may be involved in adaptive synthesis of either galacto-Waldenase or of some uridyltransferases or of a glycosyl transferring enzyme.

Kittinger & Reithel (160), who have continued their studies on lactose formation in the mammary gland (263), have obtained homogenates and a lyophilized protein fraction (precipitated with 1.5 volumes of 50 per cent ethanol) from mammary gland, both of which synthesize appreciable amount of lactose from glycogen and α -glucose-1-phosphate, whereas free glucose is not converted. The mechanism of the formation of the β -galactosidic linkage is still obscure. It could be a nonphosphorolytic transglycosidic reaction directly from starch or glycogen (presumably using the 1-4 linkages) or phosphorolytic, from α -glucose-1-phosphate. The latter is less likely since glucose-1-phosphate is not active in the absence of starch. The authors have findings which might indicate that both starch and α -glucose-1-phosphate are involved in lactose synthesis and that α -glucose-1-phosphate is involved in formation of the galactose moiety of lactose.

DPN transglycosidase.—Zatman, Colowick & Kaplan (332) have published in more detail their important work on the spleen DPN trans-N-glycosidase. They added C^{14} -labelled nicotinamide to DPN in the presence of the enzyme. After an incubation of 2 to 3 hr., only 30 per cent of the DPN was hydrolyzed, but the radioactivity per mole was now the same in the bound nicotinamide of DPN as in the free, i.e., 100 per cent exchange. The radioactive DPN was obtained in excellent yield by elution from Dowex-1-formate or by paper chromatography. In the Dowex chromatography DPN

is eluted some time after infusion with 0.1 *M* formic acid. To show that the radioactivity of the DPN was indeed located in the nicotinamide part, the DPN was hydrolyzed by the glycosidase from *Neurospora*, and the isolated nicotinamide showed the same specific activity as that of DPN.

Isonicotinic acid hydrazide (INH³) has been found to inhibit the tissue diphosphopyridine nucleotide trans-N-glycosidase [diphosphopyridine nucleotidase (331)], from a number of animal species. The species which are sensitive to INH are: beef, sheep, goat, duck, and pigeon. Among the insensitive species are man, horse, pig, rabbit, mouse, rat, and frog. The insensitive enzyme system forms and accumulates in the presence of INH an analogue of DPN where INH replaces the nicotinamide in the DPN structure. The INH analogue has been isolated and the existence of an INH riboside linkage has been proved (333). The INH analogue gives in the presence of alkali a yellow product (E_{max} 390 m). The INH analogue is not split by INH sensitive diphosphopyridine nucleotidases and is in fact a very potent inhibitor of the enzymic cleavage of ordinary DPN by this group of enzymes. It is possible that the INH analogue is also formed by the insensitive systems and that the inhibition which is observed with INH is to be attributed to a small amount of analogue synthesis which then inhibits further splitting or exchange of the DPN riboside linkage. The analogue is split by the insensitive pig brain enzyme and in the presence of the normal component, nicotinamide, DPN can be resynthesized. The diphosphopyridine nucleotidase can therefore be described as a transferase of adenosinediphosphate-ribosyl, i.e.:



Nicotinic acid hydrazide and pyrimidine-4-carboxylic acid either do not produce any analogues with insensitive enzymes or they inhibit sensitive enzymes. The diphosphopyridine from *Neurospora crassa*, which is insensitive to INH, does not yield the analogue. This enzyme is also unable to catalyze the exchange between labelled free nicotinamide and the nicotinamide of DPN. It may be of interest that extracts of mouse sarcoma and leukemic extracts from mice can catalyze the formation of the INH analogue.

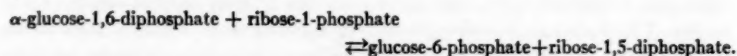
Phosphoglucomutase.—Recent work by Najjar (238) indicates that the phosphoglucomutase contains phosphate as a functional group. Highly purified enzymes seem to contain a phosphoryl group which is shed in the presence of glucose-1-phosphate. The phosphoglucomutase in tunicates has been studied by Sable & Calkins (271). Barium and magnesium ions activated this enzyme, and in the presence of versene iodoacetate had no effect on the activity. The Michaelis constants for α -glucose-1-phosphate and for magnesium ions were determined. Posternak & Rosselet (253) have synthesized α -D-mannose-1,6-diphosphate (MDP²). They confirmed Leloir's findings

that phosphoglucomutase catalyzes the conversion of mannose-1-phosphate to mannose-6-phosphate and that this reaction is activated to the same extent by GDP^3 and MDP whether the muscle or the yeast enzyme was used. They found the phosphoglucomutase activity towards G-1-P to be as much as 200 to 7000 times greater than the activity towards M-1-P. The phosphoglucomutase reaction was activated by MDP. With the muscle enzyme acting on G-1-P the same degree of activation was obtained with the two coenzymes whereas for the yeast enzyme acting on G-1-P, GDP was 2.5 times as potent an activator as was MDP.

Brown (36) found that glucosamine-6-phosphate can be enzymatically converted into the corresponding 1-ester. The equilibrium was in general more favorable towards formation of the 1-ester than in the glucose system. Almost 20 per cent was glucosamine-1-phosphate. Glucosamine-1-phosphate is also more stable than the corresponding glucose analogue; 30 min. at 100°C . in 1 N H_2SO_4 are required for complete splitting. Leloir & Cardini (193) report that N-acetyl glucosamine phosphate can also be enzymatically converted to a 1-ester; the details for this study are forthcoming.

The following 1-esters have been shown not to undergo enzymic conversion to 6-esters: β -glucose-1-phosphate, α -galactose-1-phosphate, and β -galactose-1-phosphate [see also Leloir (189)].

Phosphoribomutase.—As a close analogy to the interreaction between glucose-1,6-diphosphate and mannose phosphate (190), Klenow & Larsen (161) found that the glucose di-ester can also interact with ribose-1-phosphate. This was first revealed by the fact that α -glucose-1,6-diphosphate can act as a "co-factor" in phosphoribomutase. It is uncertain whether the latter enzyme which was isolated from muscle according to Najjar's method is identical with phosphoglucomutase. In other tissues recent findings [Guarino (111)] indicate definitely that the two enzymes are different entities since they have different properties. The muscle enzyme according to Klenow (162), catalyzes the following reaction:



The reaction can be pushed from left to right and followed spectrophotometrically by addition of glucose-6-phosphate dehydrogenase and TPN. The suspected ribose-1,5-diphosphate was isolated and purified on a Dowex-1 column and identified. The properties and composition of the isolated product was in agreement with the proposed structure. The acid lability of the 1-ester bond is not quite as high as that of ribose-1-monophosphate. It is not as yet known whether the ribose-1,5-diphosphate formed is an α or β ester. Offhand it would seem most likely that the diester as well as ribose-1-phosphate are α -esters since they can interact with α -glucose-1-phosphate and the corresponding diester. The occurrence of an α -riboside in the cell has recently been demonstrated in the case of cobalamin (vitamin B_{12}) (cf. 141). The ratio phosphoglucomutase: phosphoribomutase is about 100 in extracts from skeletal muscle. In extracts from uterus muscle the ratio is much smaller

and there are indications that there are two distinctly different enzymes [Guarino (111)].

PHOSPHATASES

UNSPECIFIC ALKALINE AND ACID PHOSPHATASES

Further evidence for the transferase activity of unspecific phosphatases has appeared in the last year. Morton (232) studied the phosphotransferase activity of alkaline phosphatase from milk and intestine. Apparently one enzyme catalyzes both hydrolysis and the transfer reaction. No enzymatic synthesis of phosphate esters could be obtained from inorganic phosphate and phosphate acceptor. Both transferase and hydrolysis activity was, however, inhibited by inorganic phosphate. By measuring the true initial rate of both hydrolysis and transfer it was found that within certain limits the transfer was increased and the hydrolysis was decreased by increasing the concentration of the phosphate acceptor. The rate of formation of dephosphorylated phosphate donor (creatine) was unchanged, however. The percentage transfer of phosphate was unaffected by the phosphate donor concentration. It was also found that both percentage transfer and the rate of the transfer reaction were independent of the donor "energy-bound." The rate of transfer is dependent only on the specificity of the enzyme. Phosphate donors are true orthophosphate monoesters or related compounds which can be hydrolyzed by the enzyme. Very similar results were obtained with acid phosphatase from human prostate. Transferase activity was found only for enzymes with low substrate specificity. The mechanism of the transferase reaction was discussed.

Another example of the transferase activity of phosphatases is given by Bravermann & Chargaff (34, 35). They found that nucleosides can be phosphorylated by a malt phosphatase or prostatic phosphatase in the presence of phenylphosphate or other nucleotides. For the prostatic enzyme system all possible nucleotides and deoxynucleotides were formed when phenylphosphate was phosphate donor. The malt enzyme catalyzed the formation of only 5-nucleotides and 5-deoxynucleotides. As phosphate donors other 5-nucleotides were superior to phenylphosphate as (see Table II).

TABLE II
ENZYMATIC PHOSPHORYLATION OF NUCLEOSIDES

Enzyme	Donor	Nucleotides Synthesized				
		5'- ribo	3'- ribo	2'- ribo	5'- deoxy	3'- deoxy
Malt	Phenylphosphate	+	—	—	+	—
	Mononucleotide	+	—	—	+	—
Prostate	Phenylphosphate	+	+	+	+	+
	Mononucleotide	—	—	—	—	—

The phosphatase content of erythrocyte hemolysate has been investigated by Tsuboi & Hudson (308). One pH optimum, around 5.5, was found with several different substrates. The hemolysate also had phosphotransferase activity. The ratio of hydrolysis to transfer was independent of the concentration of phosphate ester substrate (phenyl phosphate) where high acceptor concentration favored the transfer reaction and depressed the hydrolysis. Only one enzyme was assumed to be involved. Transferase activity has also been reported by Szulmajster *et al.* (303).

A number of papers dealing with the purification and the properties of prostatic phosphatase have appeared. A detailed investigation of the properties of the enzyme has been made by London & Hudson (206). They determined solubility and adsorption properties which suggested that it is a fairly small protein molecule. The most purified fractions were very unstable especially in the presence of large surfaces of glass and low enzyme concentrations. The presence of a nonionic surface agent (Tween 80), however, gave complete protection of enzyme activity even in the presence of a large glass surface. They obtained a 5000-fold purification of the enzyme with more than 20 per cent yield. The turn-over number was estimated to be 100,000. A significant step in the purification was foaming of the enzyme solution which under appropriate condition yielded a 16-fold purification.

Fishman & Lerner (89) have described a procedure for determination of phosphatase activity in serum. The substrate was phenyl phosphate, and the measurements depended on the determination of free phenol. The authors define "prostatic" phosphatase in serum as the acid phosphatase which is inhibited by L-tartrate. A number of normal males and male and female patients suffering from chronic diseases were found to have a ratio of total acid phosphatase to "prostatic" phosphatase from 5:1 to 10:1. In patients suffering from prostatic cancer, however, the "prostatic" phosphatase amounted to 50 to 90 per cent of the total acid serum phosphatase. In control experiments where phosphatase isolated from prostate was added to serum there was a good agreement between the amount added and the amount found by the tartrate method.

Jeffrey (137) has observed the presence of an activator for prostatic acid phosphatase in a variety of natural fluids. The activator is heat stable and is not destroyed by acid or alkali, and is not replaced by a number of metal ions. Agnostopoulos (2, 3) has studied the properties of the acid phosphatase of the prostate gland. The activating effect of citrate was most pronounced on the acid side of the pH-optimum. The optimum concentration for activation is about 10^{-2} M. Other organic acids had similar effects when an alcohol, a thiol, or a ketone group was in the α -position relative to the carboxyl. The mechanism of the activating effect of these compounds seems to be that they cause an increase in the affinity of the enzyme for the substrate. A similar effect is found on other phosphatases. The fluoride inhibition of the enzyme is counteracted by citrate. The degree of inhibition of prostatic phosphatase by L-tartrate was dependent on pH, and the inhibition was found to be com-

petitive and reversible. Several derivatives of L-tartrate seem to have little or no inhibitory effect. L-Tartrate was not inhibitory towards a number of phosphatase preparations from higher plants.

Curtois & Van Ai (71) have investigated the phosphatases of sweet chestnut leaves. The pH optimum was between 5 and 6, and the activity is scarcely influenced by the presence of Mg^{++} and Mn^{++} , whereas zinc ions inhibited the enzyme activity. At least 80 per cent of the enzyme is present in insoluble form. A number of different techniques for solubilizing the enzyme were unsuccessful. The season was found to have no influence on the properties of the enzyme. The effect of versene on phosphatases has been studied by Agnostopoulos (1). Alkaline phosphatases were completely inactivated by $2 \times 10^{-3} M$ of versene. Acid prostatic phosphatase was activated by versene which furthermore counteracts the inhibition of this enzyme by fluoride.

Harris *et al.* (112) found that the intestine bovine alkaline phosphatase can be purified by isolation and extraction of the microsomes with *n*-butanol. This fraction was further purified by acetone precipitation. A three-fold purification of renal alkaline phosphatase was obtained by Levy & Mazia (199) by electrophoresis on paper.

Roche & Bouchilloux (267) have purified alkaline intestinal phosphatase extensively by precipitation with acetone, digestion with trypsin, ammonium sulfate precipitation, and treatment with calcium phosphate gel. Paper electrophoresis of this preparation indicated three components of which only one contained phosphatase activity. The last mentioned moved as a single compound at different pH values during paper electrophoresis. The enzyme has a broad specificity and the need for a specific coenzyme could not be found.

Goldberg & Jones (103) found phosphatase from female vagina with a pH-optimum about 5. The specific activity is lower than that of the enzyme from human prostate gland, but the properties of the two enzymes are similar. The activity of the enzyme in different parts of the vagina were investigated histochemically.

Hollinger *et al.* (124) have investigated the effect of nerve section and of nerve crush on the content of phosphatases of nerves. Both treatments increased considerably the content of acid phosphatases of the nerves. It reached a maximum after 16 days and then decreased. By these treatments the alkaline phosphatase content decreased and then returned to the original or (in the case of nerve crush) to higher values, after varying time intervals. The 5'-nucleotidase activity increased after both operations and reached maximum at the thirty-second and ninety-sixth day after nerve section and nerve crush, respectively. The adenosinetriphosphatase content was uninfluenced by nerve section, but it increased and reached maximum at the ninety-sixth day after nerve crush. Changes have been found by Albaum *et al.* (4) in the content of phosphate esters of different rat tissues during storage at 18° C. After four weeks at this temperature the amount of adenine

nucleotides was appreciably decreased, especially in liver and kidney. In the case of phosphocreatine the content increased in some and decreased in other tissues but in any case these changes were rather small.

Lora-Tamayo & Baluja (208) have observed that some nucleotides inhibit renal phosphatase acting on β -glycero phosphate but not adenosine and cytidine. Bodansky (29) found that patients with high serum alkaline phosphatase activity tend also to have high serum phosphohexose isomerase activity. Studying the effect of food ingestion on alkaline phosphatases Jackson (136) found that fasting decreased the amount in serum and in intestine mucosa. After ingestion of various foods the phosphatase activity was brought back to a normal level or higher than normal. Such an effect could not be obtained by injection of glucose. This effect of food was uninfluenced by ligation of the bile duct or by introduction of a draining biliary cannula. Tuba & Robinson (305) found that the activity of alkaline phosphatase in serum and intestine of rats decreased to a certain low level after fasting. Ingestion of olive oil by the fasted animals increased the level of the enzyme activity rather quickly.

Howell & Fitzgerald (130) have investigated several species of *Actinomyces* for production of phosphatases. In these cases where phosphatase production was found the enzyme had an acid pH-optimum the value of which shifted with the temperature of growth, i.e., the pH-optimum could change from 3.0 to 4.0 by changing from 25°C. to 45°C. The total amount of phosphatase also increased under such conditions. A study of the localization of phosphatase by the Gomori technique has been performed by McGregor & Street (223) in sections of roots of tomato plants.

SPECIFIC PHOSPHATASES

Metaphosphatase.—Malmgren (215) has investigated the polymetaphosphatase activity of *Aspergillus niger*. He obtained a 20-fold purification of the enzyme by electrophoresis of the ammonium sulfate-fractionated enzyme. This preparation has the same enzyme specificity as the original extract, and the phosphatase activities to a number of phosphate esters have the same solubility in ammonium sulfate solutions. The pH-optimum of the enzyme towards polymetaphosphate is, however, different from that of any other phosphate ester tested. Also with respect to heat destruction and activators (manganese) and inhibitors (fluoride, citric acid, and phloretin phosphate) the polymetaphosphatase shows distinct differences from other phosphatase activities of the enzyme preparation. The author concludes that *A. niger* contains a specific polymetaphosphatase. Bovine liver was found to contain metaphosphatase activity which unlike the *A. niger* enzyme acted only on polymetaphosphates of low molecular weight. Lindeberg & Malmgren (201) investigated the optimal growth condition of *A. niger* with regard to polymetaphosphatase production. High enzyme production was obtained by addition of Fe, Cu, and Mn. Zn, however, inhibited enzyme production. The

best nitrogen sources were nitrates or ammonium salts. Low pH of the medium stimulated enzyme production.

Krishnan & Bajaj (177) have investigated the phosphatases of the mycelium of *A. niger*. The pH-optimum of the metaphosphatase was found to be markedly different from that of the inorganic pyrophosphatase and from that of the adenosinetriphosphatase. The ratio between the latter two was independent of the stage of growth of the mold and addition of zinc ions to the medium, whereas the ratio between the activities of metaphosphatase and inorganic phosphatase increased during aging of the medium, and the metaphosphatase activity almost disappeared when the medium contained Zn^{++} . By fractionation with alcohol or acetone inorganic pyrophosphatase fractions can be obtained almost free of metaphosphatase, but here again the ratio of inorganic pyrophosphatase to adenosinetriphosphates is almost unchanged during fractionation.

Mattenheimer (218) has found that extracts of organs from man and rat are able to hydrolyze trimetaphosphate, but not tetrametaphosphate, to orthophosphate. Yeast, however, is able to hydrolyze both the trimeta- and the tetrametaphosphate. Meyerhof *et al.* (227) have studied the properties of a specific yeast trimetaphosphatase. The ΔH of enzymic hydrolysis was 19.3 ± 0.9 kcal. per mole. The ΔF of hydrolysis was estimated to be 21 kcal. per mole (i.e., the same order of magnitude as ATP-pyrophosphate). These results may have a bearing on our view of the role of metaphosphates in the cell.

Pyrophosphatases.—Kunitz (181) has crystallized inorganic pyrophosphatase from baker's yeast. The purification was achieved by ammonium sulfate fractionation, adsorption of impurities on calcium phosphate gel, and crystallization from alcoholic solutions by isoelectric precipitation. The yield of enzyme four times recrystallized, was 16 per cent. This represented a 143-fold purification. The enzyme had an isoelectric point at pH 4.75, and its content of tyrosin and tryptophane was calculated from the absorption curve. The enzyme was strictly specific for inorganic pyrophosphate. The pH optimum was between 6 and 7 depending on the ratio of Mg to PP_i . Mg. is an activator as is also Co and Mn; the two latter, however, at the same time counteract the activation caused by Mg. Ca^{++} inhibits the effect of all three. The enzyme reaction proceeds at zero order until the reaction is almost complete. Schachman (278) studied the crystalline inorganic pyrophosphatase by means of the ultracentrifuge and by electrophoresis and found that 90 per cent of the protein was attributable to a single component. The anhydrous molecular weight was determined to be 60,000. Gilmour & Calaby (102) have described an inorganic pyrophosphatase from insect muscles. A considerably higher activity was found in the flight muscles than in the femoral muscle. The enzyme is activated by Mg^{++} , has a pH-optimum at 8.3, and seems to be specific for inorganic pyrophosphate. Norberg (242) has studied the activity of some pyrophosphatases and of adenylypyrophosphatase in the re-

generated rat liver. The activity of the enzymes studied appeared to regenerate somewhat more slowly than that of the liver mass.

Monophosphatases.—Shuster & Kaplan (279) have found an enzyme in plants with phosphatase activity specifically for the b-nucleotides of purines and pyrimidines. The enzyme was fractionated from germinating barley and from germinating rye-grass. In addition to the b-nucleotides only CoA was dephosphorylated by the enzyme which is competitively inhibited by a- and 5'-nucleotides. The pH-optimum was rather sharp at 7.5, and the Michaelis constant values for some of the substrates were determined.

In their study of the enzymatic conversion of fructose-1-phosphate Leuthardt *et al.* (197) have shown that the specific fructose diphosphatase in liver splits off the phosphate in the 1-position of the substrate. Similar results were obtained by Hers & Kusaka (118).

Cori & Green (65) have investigated the glucose-6-phosphatase activity in liver homogenates of some cases of von Gierke's disease. In all cases the activity was lower than normal. In two fatal cases it was practically absent. These findings are discussed in relation to the symptoms of the disease.

The phytase activity of wheat has been investigated by Peers (249). The pH-optimum is 5.15, and the Michaelis constant value is $0.3 \times 10^{-3} M$. The temperature optimum is at 55°C. at which temperature the enzyme is fairly heat stable. The distribution of the enzyme activity among the different anatomical parts of the wheat germ and among different species and varieties of wheat was determined.

Phosphoprotein phosphatases.—Extracts of stomach mucosa have been found by Mattenheimer (217) to contain a phosphoprotein phosphatase which is not identical with the common phosphomonoesterase of the extracts. The latter enzyme is, in contrast to the phosphoprotein phosphatase, rapidly destroyed at pH 10. The phosphoprotein phosphatase which was purified by electrodialysis was active with phosphopeptone, casein, and α -casein but was inactive with β -glycerophosphate. Several different sources of enzyme activity were found. Similarly Foote & Kind (92) found chick embryo to contain a phosphoprotein phosphatase active towards casein and phosvitin. After a 30-fold purification, the enzyme showed no activity toward a number of different phosphate esters and was therefore considered to be a true phosphoprotein phosphatase.

The dephosphorylation of ovalbumin and plakalbumin has been thoroughly studied by Perlmann (251). Prostate phosphatase dephosphorylated the A₁ component whereby the A₂ component containing only one phosphorus atom per molecule was formed. The A₂ component is dephosphorylated to the phosphorus-free A₃ component by intestinal phosphatase. In an analogous way plakalbumin was converted first to the P₂ protein by prostate phosphatase and finally to the phosphorus-free P₃ protein by intestinal phosphatase. The electrophoretic behavior of all these crystalline proteins was investigated. Prostate phosphatase was found also to dephosphorylate α -casein with a pH-optimum between 5.6 and 6.6 (250). During the dephosphoryla-

tion the solubility of the protein decreased and simultaneously several new compounds appeared, as shown by the electrophoretic pattern. The enzyme reaction was inhibited by β -casein.

A relationship between dialkylfluorophosphatase inhibition by heavy metals and formation constants of metal-amino acid complexes has been reported (233).

Phosphodiesterases.—Lundquist (210) has found that extracts of vesicular glands contain considerable amounts of glycerophosphorylcholine. The isolated compound could be converted to choline and glycerophosphate by treatment with prostata phosphatase. The formation of glycerophosphorylcholine seems to be under hormonal control since the concentration level drops considerably after castration. A similar result has been obtained independently by Diamant *et al.* (76) who identified the glycerophosphorylcholine as a ferric chloride complex.

NONPHOSPHOTRANSFERASE ENZYMES ACTING ON PHOSPHORYLATED INTERMEDIARIES OF CARBOHYDRATE METABOLISM

Transketolase.—Work by Horecker & Smyrniotis (125, 126, 128) and by Racker *et al.* (257) indicates that "active glycolaldehyde" is produced enzymatically as shown in Figure 1. The postulated active glycolaldehyde is apparently bound to the enzyme and cannot by itself be dissociated since glycolaldehyde never accumulates. Neither does free glycolaldehyde react when added to the enzyme system plus an acceptor. When various acceptor aldehydes, however, are added to the enzyme system containing a glycolaldehyde donor an accumulation of respective hydroxy acyloins takes place. The enzyme, transketolase, which catalyzes these reactions has been isolated in a highly purified state from baker's yeast (257), spinach leaves, and rat liver acetone powder (128). All these preparations contain bound thiamine-

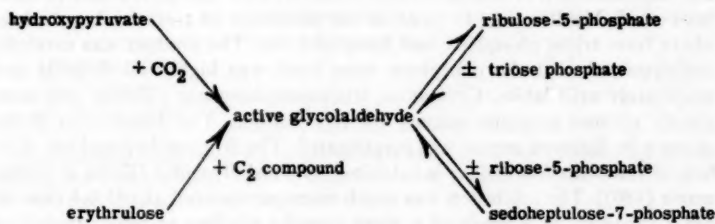
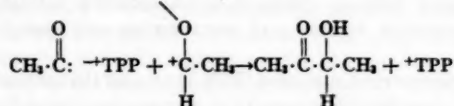


FIG. 1. Role postulated for active glycolaldehyde.

pyrophosphate which can be split from the protein either by dialysis or by ammonium sulfate precipitation at low pH. The reactions show absolute requirement for the presence of this coenzyme. There is a striking analogy between the transformation of hydroxy pyruvic acid, catalyzed by trans-

ketolase, and the formation of acyloins from α -keto acids and added aldehydes, catalyzed by carboligases. According to Reed (262) an acyloin formation may take place as follows:



It is apparent that a "phosphorylated active glycolaldehyde" ($[\text{H}_2\text{O}_3\text{-POCH}_2\text{C}(\text{O})\text{CHO}]$) produced from sedoheptulose or ribulose phosphates by trans-

ketolase or a similar enzyme may be the unidentified C_2 intermediate first recognized by Calvin and his associates (17, 45) as an initial acceptor of CO_2 during photosynthesis.

Aldolases and transaldolases.—A number of new and interesting aldolase reactions have been reported. According to Horecker & Smyrniotis (127) sedoheptulose-7-phosphate can be converted to fructose-6-phosphate in the presence of a source of aldo triosephosphate (for instance hexosediphosphate) by an enzyme purified from brewer's yeast. If the triose source is uniformly C^{14} -labelled, the newly formed hexosemonophosphate contains C^{14} in carbons 4, 5, and 6 exclusively. Carbons 1, 2, and 3 must therefore have originated from sedoheptulose-7-phosphate transferred to glyceraldehyde-3-phosphate to form fructose-6-phosphate and probably erythrose-4-phosphate.

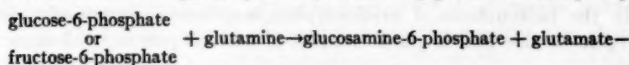
A similar reaction has been found by Hough & Jones (129) with an enzyme from peas. They isolated and characterized sedoheptulosephosphate from incubation mixtures consisting of D-erythrose, triosephosphate, and enzyme. In the presence of dihydroxymaleic acid and triosephosphate the enzyme catalyzed the formation of D-xylulosephosphate which was isolated and characterized. A soluble enzyme from rat liver has been found [Charalampous & Mueller (54)] to catalyze the formation of L-erythrulose-1-phosphate from triose phosphate and formaldehyde. The product was carefully characterized, and the phosphate ester bond was highly alkali-labile and moderately acid labile. Crystalline fructosediphosphate aldolase was completely without catalytic activity for this reaction. The distribution of the enzyme in different organs was investigated. The fructosediphosphate aldolase of *Lactobacillus bifidus* is inhibited by α , α' -dipyridyl [Kuhn & Tiedemann (180)]. The inhibition was much more pronounced at pH 6.4 than at pH 7 probably as a result of a more complex binding of heavy metals at lower pH [cf. Warburg (319a)]. The oxygen pressure had no influence on the rate of reaction which proceeded in the presence of cysteine. Therefore iron and cobalt ions, the valence of which would change under such conditions, were not considered to be activators for the enzyme. Zinc was suggested as a possible activating metal.

Isomerases.—The triosephosphate isomerase has been obtained in crystal-

line form by Meyer-Arendt *et al.* (224) free from other enzyme activities. The turnover of the enzyme was almost 1×10^6 M per min. per 10^6 gm. of protein, and the enzyme is therefore to be classified with the most active enzymes. Phosphoriboisomerase has been extensively purified from alfalfa leaves by Axelrod & Jang (10). The turn-over was about 2.7 mM per min. per mg. protein nitrogen. The enzyme has a sharp pH-optimum at pH 7 and is inhibited by *p*-chloromercuribenzoate.

Miller *et al.* (229) discuss an isomerase of the oxidation product of 1-phospho-1,2-propandiol. Glycerophosphate dehydrogenase from myogen A brings about oxidation of 1-phospho-1,2-propandiol to the corresponding keto compound. This is supposed to be converted to lactaldehydophosphate which subsequently can be oxidized to lactylphosphate. If these steps can be proved, it will be the first example in which Warburg & Christian's original suggestion for the mechanism of formation of their acylphosphate (319) found its application.

Biosynthesis of glucosamine phosphate.—Leloir & Cardini (193) have found a hitherto unrecognized pathway of making glucosamine-6-phosphate.



The reaction was observed in dialyzed enzyme preparations from *N. crassa* (juice extract from lyophilized cells fractionated with acetone). The incubate was diluted as little as possible and analyzed after 3 hr. incubation (see Table III). Whereas the production of free ammonia is about the same in all three cases the formation of glucosamine is equimolar with the consumption of amide. The suspected glucosamine-6-phosphate was compared

TABLE III
RELATION BETWEEN GLUCOSAMINE FORMATION AND AMIDE CONSUMPTION*

	Δ Glucosamine	Δ Amide	Δ NH ₃	Δ Glutamate
Complete mixture	0.42	-0.39	0.17	0.36
No glutamine	0.03	-0.07	0.14	0.08
No hexose-6-phosphate	0.03	-0.04	0.22	0.10

Experimental conditions: 1.3 M hexose-6-phosphate; 0.6 mg. fractionated enzyme preparation; citrate buffer pH 6.4, total volume; 0.1 ml. Incubation 3 hr. at 30°C.

* From Leloir & Cardini (1953).

with an authentic sample obtained by phosphorylation of glucosamine from ATP in the presence of hexokinase. Both esters were dephosphorylated before they were put on paper for chromatography and R_F values for the two products were identical. The authors emphasize the importance of separating the amide-transaminating enzyme from isomerase in order to learn whether the aldo- or the keto-hexose is the primary acceptor. It may well be that the

isomerase is essential for yielding glucosamine-6-phosphate even though fructose-6-phosphate is the primary acceptor, i.e., an imino analogue being the primary product undergoes enzymic isomerization much like the ketose.

The crude *Neurospora* preparation apparently also contains an acetylating system (cf. 55) since addition of acetate, ATP, and CoA in the presence of glucosamine gives acetyl glucosamine-6-phosphate presumably through acetylation of the 6-ester.

In the presence of glucose-1,6-diphosphate the corresponding acetyl glucosamine-1-phosphate was formed just as the nonacetylated compound can undergo the same conversion [(36); see section on phosphomutases]. In the enzymic synthesis of the glucosamine esters only glutamine is active, asparagine being unable to replace as a donor. The formation of glucosamine-6-phosphate should also be considered as a pathway in the biosynthesis of purine ribosides following the pathway described by Horecker (125) and others; the more so since the nitrogen of the N-ribosidic linkage in purine ribosides is derived specifically from the amide nitrogen of glutamine. Many of the step reactions described by Leloir & Cardini could obviously play a role in the biosynthesis of uridinediphosphoglycosyl compounds carrying acetylglucosamine and in the formation of various polymers of sugar compounds.

NEW COMPOUNDS

Chemical synthesis of diester model substances (37) has given important clues as to the mode of action of ribonuclease. The chemical structure of *a* and *b* nucleotides was finally established (38). A number of hitherto unrecognized phosphorus compounds have been isolated and identified. ATP preparations from different sources have been found to contain several other nucleotide polyphosphates. Thus, Marrian (216) found a preparation from ox-muscle to contain a substance which tentatively has been identified as adenosine-5'-tetraphosphate. Although no trace of the substance could be found in TCA³ extract of rat viscera the possibility exists that the tetranucleotide phosphate may be a naturally occurring nucleotide of unknown significance. Bergkvist & Deutsch (21) found ATP from rabbit muscles to contain 2 to 4 per cent of other triphosphates. These were isolated by ion exchange chromatography and identified by filter paper chromatography as a mixture of guanosinetriphosphate and uridinetriphosphate.

This compound has also been isolated from yeast (205) by means of ion exchange chromatography. The compound was carefully characterized with regard to acid lability, mobility on the paper chromatogram, degradation products, etc. The significance of uridinetriphosphate in several enzyme systems has already been mentioned.

Strominger (296, 297) has developed a quantitative method of measuring the accumulation of derivatives of uridine pyrophosphate N-acetylhexosamine in *Staphylococcus aureus* inhibited by penicillin and other antibiotics. By means of ion exchange chromatography at least one hitherto unidentified

compound has been isolated. The incorporation of uracil-2-C¹⁴ into nucleic acid was inhibited by penicillin and resulted in the accumulation of C¹⁴-labelled uridinepyrophosphate derivatives.

Hurlbert (132) has injected 6-C¹⁴ orotic acid into rats and found that most of the activity of perchloric acid extract to be present in uridine-5-phosphate or its derivatives. The U-5-P compounds were in close equilibrium with regard to specific activity whereas the specific activity of the uridylic acid from the nuclear RNA was only about one-half as high, suggesting that U-5-P compounds are among the immediate precursors of nucleic acid uracil.

Christie *et al.* (56) have prepared diadenosine-5'-pyrophosphate and diuridine-5'-pyrophosphate by a novel type of chemical synthesis (156). The compounds were rather stable to acid and alkali, but in the presence of crude snake venom they were hydrolyzed to the corresponding nucleosides and orthophosphate. The type specific substance of *Hemophilus influenza* which has been thoroughly studied by Zamenhof *et al.* (330) seems to consist of a poly-ribophosphate chain similar to that in pentose nucleic acids. In the *H. influenza* substance, however, the place of the purines and pyrimidines is occupied by a second chain, linked to the first on a 1:1'-glycosidic linkage. In view of the trehalose type of glycosyl linkage, the possible role of uridine polyphosphate (cf. 191) should be considered in the synthesis of this capsular substance.

The occurrence of a pyridine nucleotide not identical with DPN and TPN has been reported (283).

The phosphorylated seven carbon compound, 2-phospho-4-hydroxy-4-carboxy-adipic acid, has been shown to be an intermediate metabolite [Umbreit (309)] by the incorporation of P³². The compound is formed in *E. coli* on addition of pyruvate and a dicarboxylic acid such as fumaric or oxaloacetic acids. This coupled phosphorylation is markedly inhibited by streptomycin.

METHODS

Phosphatases and adenosinetriphosphatases.—Calcium phosphate as an indicator of the true site of alkaline phosphatase within the cell as determined in the Gomori-Takamatsu test has been considered by the Linderström-Lang group (51, 138, 139) and by Danielli (72). The former base their arguments on experiments on supersaturation of calcium phosphate and assume a theoretical model which within reason gives the most favorable conditions for the precipitation test. They find that the very fast diffusion of the phosphate from the enzymic site together with the tendency of calcium phosphate to form supersaturated solutions, makes improbable the local intracellular precipitation of phosphate in or near the enzymic site. Even a well defined distribution of solid particles according to concentration gradients seems unlikely. Danielli's experiments on spontaneous precipitation of calcium phosphate in cells devoid of active enzyme are partially in agreement with these conclusions. However, by a technique of superimposed sections and precipitation of the alcohol moiety of the phosphate esters to study

the diffusion of calcium phosphate and phosphatase, he concludes that fairly accurate localization of enzyme can be determined even within a nucleus. It seems, however, that only the intercellular and not the intracellular diffusion of calcium phosphate has been investigated by his methods.

A differential spectrophotometric method has been developed by Brandenberger & Hanson (33) for the determination of the activity of alkaline and acid phosphatases. It is based on the difference in ultraviolet absorption of *o*-hydroxybenzoic acid and of the phosphate ester of this compound. The course of the phosphatase reaction is followed by measuring the increase in absorption at 298 μ .

Green & Mommaerts (107) have worked out an electrotitrimetric method for the measurement of the adenosinetriphosphatase activity of myosin. The number of equivalents of acid generated per mole of inorganic phosphate liberated is given as a function of pH. No influence on this ratio by substrate and enzyme (myosin) concentration was observed. The equivalent ratio was found to be unchanged during the enzymatic reaction, and the influence of KCl and CaCl_2 on the equivalence curve was determined.

Determination of phosphate compounds.—Lee & Eiler (186) have studied the exchange of phosphate in acceptor-free system, where the exchange between inorganic phosphate and labile phosphate of ATP in rat brain homogenate during oxidation of substrate was followed by using P^{32} . They found that by employing the method of Le Page (194 a) for precipitation of inorganic phosphate a coprecipitation of the labile phosphate of ATP occurred. A suitable method was developed by addition of reduced quantities of magnesium to a neutralized trichloroacetic filtrate, resin-treated (in order to remove magnesium) and made 1.5 *N* with ammonium hydroxide.

Another method for determination of inorganic phosphate in the presence of readily hydrolyzable phosphate compounds is described by Chambers & Mende (52). This is particularly suitable to systems where turbid extracts must be used and is a modification of the Berenblum & Chain (18) procedure based on the formation of a phosphomolybdic acid complex in isobutyl-alcoholic extract.

Slater (287) has adapted Racker's method (256) for determination of hexosediphosphate, adenosinetriphosphate, adenosinediphosphate, glucose-6-phosphate, glucose-1-phosphate, and fructose-6-phosphate and elaborated it further since the preliminary report (285). The method depends on the formation of dihydroxyacetonephosphate by specific enzymes and determination of the dihydroxyacetonephosphate by following the oxidation of reduced diphosphopyridine nucleotide in the presence of glycerol phosphate dehydrogenase. Separate analyses were obtained for four groups of phosphorus containing compounds: (a) Hexosediphosphate and triose phosphates; (b) hexosemonophosphates: glucose-1-phosphate, glucose-6-phosphate, fructose-1-phosphate (not free fructose-6-phosphate); (c) adenosine triphosphate; (d) adenosinediphosphate, creatinephosphate, phosphopyruvate. Differ-

ential values for the adenosinetriphosphate and creatinephosphate in the last group were obtained by Kratzing & Narayanaswami (175) by enzymatic determination of the adenosinediphosphate with adenylate kinase.

Beisenherz *et al.* (15) have carefully worked out detailed conditions for the preparation of five different crystalline metabolic significant enzymes from a single batch of rabbit muscle extract. Many of the enzymes described are obtained in high purity and in a more convenient way than have earlier been described.

For the separation of nucleotides from other phosphate esters and orthophosphate, norite is very useful. The nucleotides are adsorbed to the charcoal at acid pH and can be recovered again by elution with alcohol in contrast to sugar phosphate ester, orthophosphate, and pyrophosphate. This principle was used by Kalckar & Cutolo (149) in their study on pyrophosphorolysis of uridinediphosphate glucose and by Crane & Lipmann (68) in their work on aerobic phosphorylation.

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